



THE THIRD EUROPEAN

**INFLUENZA**  
**CONFERENCE**

VILAMOURA | PORTUGAL | 14-17 SEPTEMBER 2008

## Programme and Abstract Book



Organised by the European Scientific Working group on Influenza

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## MESSAGE FROM THE ESWI PRESIDENT

Following the success of the two previous conferences and in response to the high level of interest shown, I now have the pleasure to welcome you to the Third European Influenza Conference, organized by the European Scientific Working group on Influenza (ESWI).

To keep you on top of the latest developments in the rapidly evolving field of influenza, the conference programme encompasses every aspect of influenza prevention, control and treatment. The twelve sessions and the various workshops and plenary lectures of the scientific programme track will provide you with state-of-the-art knowledge on influenza as well as a forum for exchanging ideas and new research strategies.

Beyond science are the public health burdens of epidemic influenza and the potentially devastating impacts of pandemic influenza. Communication and cooperation between scientists, policy makers and healthcare professionals is key to significantly reducing the burden of influenza. The conference therefore offers clearly delineated sessions and workshops for government representatives and opinion leaders in healthcare (Science In Practice track).

ESWI also supports young scientists in their careers and their research which looks to advance what we know about influenza. Through the Young Scientist Fund, ESWI has provided some 50 grants to young scientists. Successful grant applicants will play an active role in the conference, each presenting their research either orally or in poster format. In fact, Tuesday 16 September will open with a plenary session entirely devoted to the work of promising scientists. In addition, young scientists have been invited to act as co-chairs during the conference sessions.

The Organizing Committee is convinced that the conference programme will encourage a high level of interaction among representatives of various disciplines. The combination of expert opinions, scientific evidence, peer contributions and active participant involvement will ensure we achieve the ESWI goal: to identify and communicate with stakeholders and provide them with opportunities for interaction.

On behalf of ESWI, I would like to thank everyone who has contributed to the preparations for this conference, with a special word of thanks to the members of the Programme Committee for their tireless efforts in organizing the scientific programme, and also to the session chairs for reviewing over 350 abstracts. Finally, my sincere thanks go to the sponsoring partners for backing such an important issue.

I wish you all an enjoyable, productive and educational meeting.

Prof. Dr. A.D.M.E. Osterhaus,

ESWI president  
Conference chair

# TOGETHER, WE CAN ACHIEVE BETTER FLU PROTECTION.

## The drive for better flu protection.

Influenza puts millions of lives at risk, each year<sup>1</sup>. Since 1968, influenza immunization has made giant steps forward, but we must all continue to focus our efforts on meeting the World Health Organization's 2010 objective of a 75% coverage rate in high-risk populations<sup>2</sup>.



AT THE FOREFRONT OF  
INFLUENZA PROTECTION

## A joint force to protect patients.

Every patient at risk deserves protection against flu. As a Healthcare Professional or a Policy Maker, you are a key actor in the prevention of flu.

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We have been at the forefront of influenza protection since the launch of Vaxigrip in 1968<sup>3</sup>. Today, Sanofi Pasteur MSD's flu vaccines are the most widely used in Western Europe, and we are exploring new approaches to further enhance protection against flu. Our experience and technologies are at the service of future progress in influenza immunization.

(1) Ryan J, Zoellner Y, Gradil B, et al. Establishing the health and economic impact of influenza vaccination within the European Union 25 countries. *Vaccine* 2006;24(47-48): 6812-22.  
(2) World Health Organization. Prevention and control of influenza pandemics and annual epidemics. Resolution of the World Health Assembly, 10<sup>th</sup> plenary meeting, Geneva 2003; WHA56.19.  
(3) Vaxigrip European SmPC 2007.

## COMMITTEES

### ORGANIZING COMMITTEE

- Dr. Ab Osterhaus  
*Erasmus MC, The Netherlands (conference chair)*
- Dr. Ted van Essen  
*University Medical Center Utrecht, The Netherlands*
- Dr. Derek Smith  
*University of Cambridge, United Kingdom*
- Dr. Francesco Blasi  
*European Respiratory Society, Italy*
- Dr. Karl Ekdahl  
*European Centre for Disease Prevention and Control, Sweden*
- Dr. Claude Hannoun  
*Paris, France*
- Dr. Raul Amaral-Marques  
*Lisbon, Portugal*
- Dr. Bram Palache  
*Weesp, The Netherlands*
- Mrs. Chris Vanlangendonck  
*Link Inc, Communication Consultants, Belgium*

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- Dr R. Amaral-Marques, Portugal  
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Dr W. Haas, Germany  
Dr C. Hannoun, France  
Dr F. Hayden, Switzerland  
Dr T. Heikkinen, Finland  
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Dr R. Krug, USA  
Dr R. Lamb, USA  
Dr J. McElhaney, USA  
Dr A. Monto, USA  
Dr P. Openshaw, UK  
Dr A. Osterhaus, The Netherlands  
Dr B. Palache, The Netherlands  
Dr P. Palese, USA  
Dr M. Peiris, Hong Kong  
Dr R. Prymula, Czech Republic  
Dr C. Russell, UK  
Dr D. Smith, UK  
Dr D. Swayne, USA  
Dr T. Szucs, Switzerland  
Dr S. van der Werf, France  
Dr J. Wood, UK  
Dr J. Yewdell, USA

## ESWI

The Third European Influenza Conference is organized by the European Scientific Working group on Influenza (ESWI). This multidisciplinary group of scientists aims to combat the epidemic and pandemic impact of influenza. More information on ESWI can be found on [www.eswi.org](http://www.eswi.org) or through ESWI's management at Link Inc, communication consultants, Mr. David De Pooter, Tolstraat 9, 2000 Antwerp, Belgium, phone +32 3 232 93 42, fax + 32 2 232 17 04 or [info@linkinc.be](mailto:info@linkinc.be)

The proceedings of the Third European Influenza Conference will be published in a dedicated issue of Vaccine in March 2009. All registered participants will receive a free copy. Prior to that, the proceedings will be made available in an online open access module.



# Influenza: navigating the winds of change with antiviral therapy

Satellite symposium  
Third European Influenza Conference

Monday 15 September 2008, 18.00–19.30  
Tivoli Marina Vilamoura Hotel, Vilamoura, Portugal

## Faculty

Robert Booy (Australia)  
Bruno Lina (France)  
Jonathan Van Tam (UK)  
John Watkins (UK)

## Roche exhibition booth

Stand number 1, Winter Garden Lobby, Tivoli Marina  
Vilamoura Hotel



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## CME ACCREDITATION

The Third European Influenza Conference is accredited by the European Accreditation Council for Continuing Medical Education (EACCME) to provide a maximum of 21 hours of European external CME credits. All medical specialists are invited to claim those hours of credit that he/she spent in the educational activity.

In line with the guidelines of CME accreditation, ESWI is keen to sustain audience confidence in the academic presentations by having lecture content that is valid and free from commercial bias. ESWI strongly believes such an approach is aligned with the best interests of the conference attendee and enforces transparency, both of which are key principles of ESWI.

ESWI therefore requested that all invited speakers disclose any relationship with a commercial interest if both (a) the relationship is financial and has occurred within the past 12 months and (b) the invited speaker has the opportunity to affect the lecture content about the products or services of that commercial interest.

A panel of commercially disinterested peers had been tasked with ensuring that all content is valid and free from commercial bias, references the best available evidence and is aligned with the interest of the conference attendee. In order to alert the attendee of the potential for conflict of interest, disclosure information will feature on a slide preceding the formal presentation of the invited speaker.



Nobilon, part of Schering-Plough Corporation, is a young biotechnology company dedicated to develop and produce vaccines against infectious diseases in humans. The R&D program includes vaccines against Influenza, Respiratory Syncytial Virus (RSV), Traveller's diarrhea, and Chlamydia trachomatis.

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# FULL PROGRAMME

## PROGRAMME SCIENTIFIC SESSIONS

14 September 2008		
13h15-14h45	SATELLITE SYMPOSIUM	FENIX I-III
– GSK Biologicals		
15h00-16h30	SATELLITE SYMPOSIUM	FENIX I-III
– Solvay Biologicals		
16h45-18h15	SATELLITE SYMPOSIUM	FENIX I-III
– Baxter		
18h30-19h30	OPENING OF THE CONFERENCE	FENIX I-III
– keynote lecture by Dr R. Anderson, <i>Imperial College of London, UK</i> : Plagues and People: Planning for Pandemics		
20h30-23h00	CONFERENCE WELCOME DINNER	

15 September 2008		
7h00-8h30	SATELLITE SYMPOSIUM	FENIX I-III
– Infectious Diseases Society of Finland, supported by MedImmune		
8h45-10h00	PLENARY 1	FENIX I-III
– <b>PL01-1</b> keynote lecture by Dr P. Palese, <i>Mount Sinai School of Medicine, USA</i> : Genes contributing to the pathogenicity of pandemic influenza viruses		
– <b>PL01-2</b> keynote lecture by Dr A. Monto, <i>University of Michigan, USA</i> : Prepandemic vaccines: yes or no?		
10h00-10h30	COFFEE BREAK	
10h30-12h00	SESSION 1 SS01	NEPTUNO

### Virus host interaction/pathogenesis/transmission

#### CHAIR:

– Dr A. Garcia-Sastre, *Mount Sinai School of Medicine, USA*

#### CO-CHAIR YS:

– Dr G. Conenello, *Mount Sinai School of Medicine, USA*

#### DISCUSSANTS:

- **SS01-1** Dr A. Garcia-Sastre, *Mount Sinai School of Medicine, USA*: Interferon antagonist functions of the NS1 protein of influenza virus
- **SS01-2** Dr Y. Kawaoka, *University of Wisconsin School of Veterinary Medicine, USA*
- **SS01-3** Dr T. Tumpey, *Centers for Disease Control, USA*: Mutations that affect the transmissibility of influenza A viruses
- **SS01-4** Dr T. Kuiken, *Erasmus MC: Pathology of influenza in humans revisited*
- **SS01-5** Dr G. Conenello, *Mount Sinai School of Medicine, USA*: Increased Pathogenesis by influenza A virus expressing a PB1-F2 protein with the N66S mutation: role of CD8+ and CD4+T-cells

### SESSION 2 SS02

FENIX I-III

### Clinical impact & diagnostics approaches

#### CHAIR:

– Dr F. Hayden, *World Health Organisation, Switzerland*

#### CO-CHAIR YS:

– Dr J. Kovar, *University College London, UK*

#### DISCUSSANTS:

- **SS02-1** Dr T. Heikkinen, *University of Turku, Finland*: Disease burden and diagnosis of influenza in children
- **SS02-2** Dr K. Nicholson, *University of Leicester, UK*: Burden of influenza and other respiratory viruses in a University Hospital setting at the extremes of life
- **SS02-3** Dr M. De Jong, *The Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam*: Human H5N1 disease: pathogenesis and treatment
- **SS02-4** Dr M. Rothberg, *Tufts University, USA*: Mortality Benefits of Statins for Pneumonia when Influenza is Circulating

### 12h00-13h30 LUNCH

### SATELLITE SYMPOSIUM

FENIX I-III

– European Vaccine Manufacturers

### 13h30-15h00 SESSION 3 SS03

NEPTUNO

### Virus structure & replication

#### CHAIR:

– Dr R. Lamb, *Howard Hughes Medical Institute, USA*

#### CO-CHAIR YS:

– Dr E. De Wit, *Department of Virology, Erasmus MC, The Netherlands*

#### DISCUSSANTS:

- **SS03-1** Dr R. Krug, *University of Texas, USA*: Multiple Functions of the NS1 Protein of Influenza A Viruses
- **SS03-2** Dr R. Lamb, *Howard Hughes Medical Institute, USA*: Influenza Virus Budding
- **SS03-3** Dr N. Naffakh, *Institut Pasteur, France*: The influenza virus RNA polymerase: a key determinant for viral host-range and pathogenicity, a target for antiviral strategies
- **SS03-4** Dr E. Carnero, *Mount Sinai School of Medicine, USA*: Nucleotides outside the predicted packaging sequences of the HA segment of influenza A virus influence the incorporation of this segment into virions

**Vaccines: current and novel approaches****CHAIR:**– Dr A. Osterhaus, *Erasmus MC, The Netherlands***CO-CHAIR YS:**– Dr J. Steel, *Mount Sinai School Of Medicine, New York, USA***DISCUSSANTS:**– **SS04-1** Dr M. Tashiro, *National Institute of Infectious Diseases, Japan*: The TLR3 agonist, PolyI:PolyC12U, added to Influenza vaccines as a nasal adjuvant induces a wide spectrum cross-protection against different subtypes including highly pathogenic H5N1 avian influenza virus– **SS04-2** Dr W. Fiers, *Ghent University, Belgium*: A Universal Human Influenza A Vaccine– **SS04-3** Dr T. Vesikari, *University of Tampere Medical School, Finland*: MF59™ Adjuvanted Influenza Vaccine (Fluad®) in Children: Safety and Immunogenicity Following a Second Year Seasonal Vaccination– **SS04-4** Dr J. Kreijtz, *Erasmus MC Department of Virology, The Netherlands*: Recombinant modified vaccinia virus Ankara expressing HA confers protection against homologous and heterologous H5N1 influenza virus infections in macaques**15h00-15h30** COFFEE BREAK**15h30-17h00** SESSION 5 SS05

FENIX I-III

**Late Breakers****CHAIR:**– Dr J. Katz, *Centers for Disease Control and Prevention, USA***CO-CHAIR YS:**– Dr A. Lowen, *Mount Sinai School of Medicine, USA***DISCUSSANTS:**– Dr Boettcher, *Institute of Virology, Philipps University Marburg, Germany*: Membrane-associated human airway trypsin-like protease (HAT) cleaves the influenza virus hemagglutinin at the cell surface

SESSION 6 SS06

NEPTUNO

**How to evaluate vaccine effectiveness?****CHAIR:**– Dr A. Monto, *University of Michigan, USA***DISCUSSANTS (PANEL DISCUSSION):**– **SS06-1** Dr L. Simonsen, *National Institutes of Health, USA*: Influenza Vaccination and Mortality Benefits: New Insights, New Opportunities– **SS06-2** Dr K. Nichol, *Minneapolis VA Medical Center, USA*: Challenges in evaluating influenza effectiveness– **SS06-3** Dr E. Hak, *University Medical Center Utrecht, The Netherlands*: Impact of influenza vaccination on mortality risk among the elderly: methodological inquiry– **SS06-4** The Cochrane Collaboration (to be confirmed)**17h00-18h00** POSTER SESSION

BLUE CORNER

WINTER GARDEN

**18h00-19h30** SATELLITE SYMPOSIUM

FENIX I-III

– F. Hoffmann-La Roche

**16 September 2008****7h00-8h30** SATELLITE SYMPOSIUM

FENIX I-III

– Sanofi Pasteur MSD

**8h45-10h00** PLENARY 2

FENIX I-III

**CHAIR:**– Dr C. Russell, *University of Cambridge, UK***CO-CHAIR:**– Dr C. Hannoun, *ESWI Young Scientist Fund***LECTURES BY ESWI YOUNG SCIENTISTS AWARD WINNERS**– **PL02-1** Dr C. Russell, *University of Cambridge, UK*: The Global Circulation of Seasonal Influenza A (H3N2) Viruses– **PL02-2** Dr A. Lowen, *Mount Sinai School of Medicine, USA*: Influenza virus transmission: studies in the guinea pig model– **PL02-3** Dr J. Schneider, *Robert Koch Institute, Germany*: The nonstructural NS1 protein of influenza B virus interacts with nuclear speckle domains– **PL02-4** Dr E. Hutchinson, *University of Cambridge, UK*: Genetic analysis of cis-acting RNA sequences in influenza A– **PL02-5** Dr K. Grebe, *NIH, USA*: The Sympathetic Nervous System Modulates Anti-Influenza CD8+ T cell Responses in vivo– **PL02-6** Dr J. McAuley, *St Jude Children's Research Hospital, USA*: The 1918 Influenza A Virus PB1-F2 Protein Contributes to the Immunopathogenesis of Viral and Secondary Bacterial Pneumonia**10h00-10h30** COFFEE BREAK

**Antivirals and resistance****CHAIR:**– Dr R. Krug, *University of Texas, USA***CO-CHAIR YS:**– Dr M. Rameix-Welti, *Institut Pasteur, France***DISCUSSANTS:**

- **SS07-1** Dr W. DeGrado, *Department of Biochemistry and Biophysics, School of Medicine, University of Pennsylvania, USA*: How recently published structure of the M2 ion channel protein provides insights for developing new antivirals against this protein
- **SS07-2** Dr A. Hay, *National Institute of Medical Research, UK*: The Structural Basis of Neuraminidase Inhibitor Resistance
- **SS07-3** Dr M. Zambon, *Health Protection Agency, UK*: Oseltamivir Resistant Influenza A H1N1 Viruses
- **SS07-4** Dr M. Rameix-Welti, *Institut Pasteur, France*: Enzymatic properties of the neuraminidase of seasonal H1N1 influenza viruses provide insights for the emergence of natural resistance to oseltamivir
- **SS07-5** Dr O. Ferraris, *CNRS, France*: Multiple detection of influenza A/H3 viruses lacking the Na gene segment

**SESSION 8** SS08**NEPTUNO****Genetic and antigenetic evolution****CHAIR:**– Dr N. Cox, *Centers for Disease Control and Prevention, USA***CO-CHAIR YS:**– Dr R. Garten, *Influenza Division, Centers for disease control and prevention, USA***DISCUSSANTS:**

- **SS08-1** Dr A. Klimov, *Centers for Disease Control and Prevention, USA*: Evolution of Influenza Viruses and Vaccine Strain Selection Process
- **SS08-2** Dr B. Koel, *Department of Virology, Erasmus MC, The Netherlands*: Mapping the molecular determinants of antigenic evolution of the influenza A (H3N2) virus
- **SS08-3** Dr N. Lewis, *University of Cambridge, UK*: The antigenic and genetic evolution of equine influenza virus (h3n8) from 1976 to 2007
- **SS08-4** Dr J. Bollback, *University of Edinburgh, UK*: Reassortment and recombination in influenza
- **SS08-5** Dr E. Fournier, *CNRS, France*: Influenza A packaging control by RNA/RNA interactions: identification of critical domains also controlling genetic reassortment

**12h00-13h30 LUNCH****LUNCH SESSION****NEPTUNO**

- **LUSS-1** Dr C. Schmaltz, *European Commission, Research Directorate General*: How far is it to Brussels? - Your voice in EU research funding for influenza –
- **LUSS-2** Dr J. Serratos, *European Food Safety Authority (EFSA), Animal Health and Welfare (AHAW)*: High Pathogen Avian Influenza: EFSA's evaluation of risks in Animal Health

**13h30-15h00 SESSION 9** SS09**FENIX I-III****Animal flu-ecology****CHAIR:**– Dr R. Fouchier, *Erasmus MC, The Netherlands***CO-CHAIR YS:**– Dr N. Lewis, *University of Cambridge, UK***DISCUSSANTS:**

- **SS09-1** Dr D. Suarez, *USA Department of Agriculture, USA*: Avian Influenza in the Live Bird Markets in the U.S.
- **SS09-2** Dr R. Webster, *St. Jude Children's Research Hospital, USA*: The role of migratory birds in the origin and perpetuation of influenza viruses
- **SS09-3** Dr V. Munster, *Department of Virology, Erasmus MC, The Netherlands*: Avian influenza virus; virus and bird ecology
- **SS09-4** Dr I. Brown, *Veterinary Laboratory Agency, UK*: Multiple incursions of H5N1 highly pathogenic avian influenza virus in to Europe
- **SS09-5** Dr K. Van Reeth, *Ghent University, Belgium*: Immunity to H1N1 swine influenza virus can partially protect pigs against a low pathogenic H5N1 avian influenza virus

**SESSION 10** SS10**NEPTUNO****Mathematical modeling****CHAIR:**– Dr D. Smith, *University of Cambridge, UK***CO-CHAIR YS:**– Dr W. Alonso, *Fogarty International Center- National Institutes of Health, Florianopolis, Brazil***DISCUSSANTS:**

- **SS10-1** Dr P. Thomas, *St Jude Children's Research Hospital, USA*: CD8 T cell epitope recognition: broad detection through a narrow window
- **SS10-2** Dr I. Longini, *Fred Hutchinson Cancer Research Center, USA*: Mathematical Modeling of Pandemic Influenza Containment and Control
- **SS10-3** Dr S. Cauchemez, *MRC Centre for Outbreak Analysis and Modelling, Department of Infectious Diseases Epidemiology, Imperial College London, UK*: Estimating the impact of school closure on influenza transmission from Sentinel data
- **SS10-4** Dr C. Viboud, *Fogarty International Center, National Institutes of Health, Bethesda, USA*: Extreme risk of influenza death among young adults: A comparative modeling study of the 1918-20 pandemic in 13 countries
- **SS10-5** Dr B. Cowling, *University of Hong Kong, Hong Kong*: Estimating the serial interval of influenza from natural infections in households

**15h00-15h30 COFFEE BREAK**

<b>15h30-17h00</b>	<b>SESSION 11</b> SS11	<b>NEPTUNO</b>
<b>Immunology</b>		
<b>CHAIR:</b>		
– Dr J. Yewdell, <i>NIAID, NIH, USA</i>		
<b>CO-CHAIR YS:</b>		
– Dr A. Wahl, <i>University of Oklahoma, USA</i>		
<b>DISCUSSANTS:</b>		
– <b>SS11-1</b> Dr G. Air, <i>University of Oklahoma Health Sciences Center, USA</i> : Antibody quality versus quantity		
– <b>SS11-2</b> Dr H. Lu, <i>Influenza Division, NCIRD, CDC, USA</i> : Influenza A H1N1 neuraminidase antibodies fail to provide cross-protection against lethal challenge with a highly pathogenic avian influenza A H5N1 infection in mice		
– <b>SS11-3</b> Dr H. Golding, <i>CBER, FDA, USA</i> : Analysis of Antibody Repertoires in H5N1 Infected and Vaccinated Individuals using Influenza Whole Genome Phage Display Libraries		
– <b>SS11-4</b> Dr G. Rimmelzwaan, <i>Erasmus MC, The Netherlands</i> : Influenza virus CTL epitopes, remarkably conserved and remarkably variable		
– <b>SS11-5</b> Dr J. Yewdell, <i>NIAID, NIH, USA</i> : tRNA Misacylation: A novel Innate Immune Response to Viruses” – CTLs, Misacylation, and Supercoding		
<b>SESSION 12</b> SS12		<b>FENIX I-III</b>
<b>Vaccine evaluation</b>		
<b>CHAIR:</b>		
– Dr J. Wood, <i>National Institute for Biological Standards and Control, UK</i>		
<b>CO-CHAIR YS:</b>		
– Dr J. Kwong, <i>Institute For Clinical Evaluative Sciences, Toronto, Canada</i>		
<b>DISCUSSANTS:</b>		
– <b>SS12-1</b> Dr J. Wood, <i>National Institute for Biological Standards and Control, UK</i> : New challenges for influenza vaccine development		
– <b>SS12-2</b> Dr D. Skowronski, <i>BC Center for Disease Control, Canada</i> : Component-specific efficacy of trivalent influenza vaccine: sentinel surveillance to detect virus variation and impact on protection		
– <b>SS12-3</b> Dr D. Shay, <i>CDC, USA</i> : Multi-state Case-control Study of the Effectiveness of Influenza Vaccine in Preventing Laboratory-confirmed Influenza Hospitalizations among Children Aged 6-23 months during the 2005-06 and 2006-07 Seasons		
– <b>SS12-4</b> Dr J.C. Kwong, <i>Institute for Clinical Evaluative Sciences, Canada</i> : The effect of universal influenza immunization on mortality and health care use		
– <b>SS12-5</b> Dr N. Sugaya, <i>Keiyu Hospital, Japan</i> : Mass Vaccination of Schoolchildren and Influenza Outbreaks in a School		
<b>17h00-18h00</b>	<b>POSTER SESSION</b>	<b>BLUE CORNER WINTER GARDEN</b>
<b>18h00-19h30</b>	<b>SATELLITE SYMPOSIUM</b>	<b>FENIX I-III</b>
– Novartis		
<b>20h30-23h00</b>	<b>ESWI ANNIVERSARY DINNER</b>	

<b>17 September 2008</b>		
<b>7h00-8h30</b>	<b>SATELLITE SYMPOSIUM</b>	
– Sanofi Pasteur		
<b>8h45-10h00</b>	<b>PLENARY 3</b>	<b>FENIX I-III</b>
– <b>PL03-1</b> Keynote lecture by Dr M. Peiris, <i>University of Hong Kong, Hong Kong</i> : Avian Influenza from the South East Asian Perspective		
– <b>PL03-2</b> Keynote lecture by Dr D. Swayne, <i>Southeast Poultry Research Laboratory, USDA/Agricultural Research Service</i> : Veterinary influenza vaccines: Strategies and Challenges to the Development and Application of Avian Influenza Vaccines in Birds		
<b>10h00-10h30</b>	<b>COFFEE BREAK</b>	
<b>10h30-12h00</b>	<b>PLENARY 4</b>	<b>FENIX I-III</b>
<b>Pandemic preparedness</b>		
<b>CHAIR:</b>		
– Dr K. Fukuda, <i>World Health Organisation, Switzerland</i>		
<b>CO-CHAIR:</b>		
– Dr K. Ekdahl, <i>ECDC, Sweden</i>		
<b>DISCUSSANTS:</b>		
– <b>PL04-1</b> Dr J. Katz, <i>Influenza Division, US Centers for Disease Control and Prevention, Atlanta, Georgia, USA</i> : Assessing the Pandemic Risk of Avian Influenza Viruses		
– <b>PL04-2</b> Dr N. Ferguson, <i>University of London, UK</i> : Predicting the impact of layered interventions		
– <b>PL04-3</b> Dr J. Van-Tam, <i>University of Nottingham, UK</i>		
– <b>PL04-4</b> Dr J. Watkins, <i>University of Wales, UK</i> : Pandemic Planning at a Sub-National Level		
<b>12h00-13h00</b>	<b>CLOSING SESSION</b>	<b>FENIX I-III</b>
– Keynote lecture by Dr A. Osterhaus, <i>Erasmus MC, The Netherlands</i>		
– Awards to young scientists		
<b>13h00-14h30</b>	<b>LUNCH</b>	

# PROGRAMME SCIENCE IN PRACTICE

## 14 September 2008

**13h15-14h45** SATELLITE SYMPOSIUM

FENIX I-III

– GSK Biologicals

**15h00-16h30** SATELLITE SYMPOSIUM

FENIX I-III

– Solvay Biologicals

**16h45-18h15** SATELLITE SYMPOSIUM

FENIX I-III

– Baxter

**18h30-19h30** OPENING OF THE CONFERENCE

FENIX I-III

– keynote lecture by Dr R. Anderson, *Imperial College of London, UK*: Plagues and People: Planning for Pandemics

**20h30-23h00** CONFERENCE WELCOME DINNER

## 15 September 2008

**7h00-8h30** SATELLITE SYMPOSIUM

FENIX I-III

– Infectious Diseases Society of Finland, supported by MedImmune

**8h45-10h00** PLENARY 1

FENIX I-III

– **PL01-1** keynote lecture by Dr P. Palese, *Mount Sinai School of Medicine, USA*: Genes contributing to the pathogenicity of pandemic influenza viruses

– **PL01-2** keynote lecture by Dr A. Monto, *University of Michigan, USA*: Prepandemic vaccines: yes or no?

**10h00-10h30** COFFEE BREAK

**10h30-12h00** SESSION 1 SIP01

GEMINI I-III

### Increasing the overall epidemic vaccination coverage

CHAIR:

– Dr A. Monto, *University of Michigan, USA*

DISCUSSANTS:

– **SIP01-1** Dr D. Fedson, *France*: Increasing the overall epidemic vaccination coverage: the macroepidemiology of influenza vaccination

– **SIP01-2** Dr. T. Szucs, *Institute of Social and Preventive Medicine, University of Zurich, Switzerland*: Influenza vaccination coverage rates in four European countries during winter of 2007/08

– **SIP01-3** Dr K. Nichol, *Minneapolis VA Medical Center, USA*: Maximizing seasonal influenza vaccination coverage

**12h00-13h30** LUNCH

SATELLITE SYMPOSIUM

FENIX I-III

– European Vaccine Manufacturers

**13h30-15h00** SESSION 2 SIP02

GEMINI I-III

### Seasonal vaccination of health care workers

CHAIR:

– Dr T. van Essen, *University Medical Center Utrecht, The Netherlands*

DISCUSSANTS:

– **SIP02-1** Dr R. Jordan, *University of Birmingham, UK*: Effectiveness and cost-effectiveness of vaccinating healthcare workers against influenza, and strategies to improve uptake

– **SIP02-2** Dr H. Van Delden, *University Medical Center Utrecht, The Netherlands*: Ethics of mandatory vaccination against influenza for health care workers

– **SIP02-3** Dr D. Walter, *Robert Koch Institute, Germany*: National influenza immunization campaign – focus on health care workers

**15h00-15h30** COFFEE BREAK

**15h30-17h00** SESSION 3 SIP03

GEMINI I-III

### Epidemic and pandemic use of antivirals

CHAIR:

– Dr F. Hayden, *World Health Organisation, Switzerland*

DISCUSSANTS:

– **SIP03-1** Dr N. Ferguson, *University of London, UK*: Antiviral use in a pandemic: predicting impact and the risk of resistance

– **SIP03-2** Dr J. Van-Tam, *University of Nottingham, UK*

– **SIP03-3** Dr F. Hayden, *World Health Organisation, Switzerland*: Antivirals for Seasonal, A(H5N1), and Pandemic Influenza: Efficacy, Resistance, and New Agents

**17h00-18h00** POSTER SESSION

BLUE CORNER  
WINTER GARDEN

**18h00-19h30** SATELLITE SYMPOSIUM

FENIX I-III

– F. Hoffmann-La Roche

16 September 2008		
7h00-8h30	SATELLITE SYMPOSIUM	FENIX I-III
– Sanofi Pasteur MSD		
8h45-10h00	PLENARY 2	FENIX I-III
CHAIR: – Dr C. Russell, <i>University of Cambridge, UK</i>		
CO-CHAIR: – Dr C. Hannoun, <i>ESWI Young Scientist Fund</i>		
LECTURES BY ESWI YOUNG SCIENTISTS AWARD WINNERS		
– <b>PL02-1</b> Dr C. Russell, <i>University of Cambridge, UK</i> : The Global Circulation of Seasonal Influenza A (H3N2) Viruses		
– <b>PL02-2</b> Dr A. Lowen, <i>Mount Sinai School of Medicine, USA</i> : Influenza virus transmission: studies in the guinea pig model		
– <b>PL02-3</b> Dr J. Schneider, <i>Robert Koch Institute, Germany</i> : The nonstructural NS1 protein of influenza B virus interacts with nuclear speckle domains		
– <b>PL02-4</b> Dr E. Hutchinson, <i>University of Cambridge, UK</i> : Genetic analysis of cis-acting RNA sequences in influenza A		
– <b>PL02-5</b> Dr K. Grebe, <i>NIH, USA</i> : The Sympathetic Nervous System Modulates Anti-Influenza CD8+ T cell Responses in vivo		
– <b>PL02-6</b> Dr J. McAuley, <i>St Jude Children's Research Hospital, USA</i> : The 1918 Influenza A Virus PB1-F2 Protein Contributes to the Immunopathogenesis of Viral and Secondary Bacterial Pneumonia		
10h00-10h30	COFFEE BREAK	
10h30-12h00	SESSION 4 SIP04	GEMINI I-III
<b>Prepandemic and pandemic vaccination</b>		
CHAIR: – Dr D. Smith, <i>University of Cambridge, UK</i>		
DISCUSSANTS (PANEL DISCUSSION):		
– <b>SIP04-1</b> Dr B. Schwartz, <i>National Vaccine Program Office, USA</i> : Prioritizing pandemic influenza vaccination: public values and public policy		
– <b>SIP04-2</b> Dr I. Longini, <i>Fred Hutchinson Cancer Research Center, USA</i> : Strategies for Containing or Slowing the Spread of Pandemic Influenza		
– <b>SIP04-3</b> Dr A. Osterhaus, <i>Erasmus MC, The Netherlands</i> : Pandemic vaccination: constraining a moving target		
12h00-13h30	LUNCH	
LUNCH SESSION		FENIX I-III
– <b>LUSS-1</b> Dr C. Schmaltz, <i>European Commission, Research Directorate General</i> : How far is it to Brussels? - Your voice in EU research funding for influenza –		
– <b>LUSS-2</b> Dr J. Serratos, <i>European Food Safety Authority (EFSA), Animal Health and Welfare (AHAW)</i> : High Pathogen Avian Influenza: EFSA's evaluation of risks in Animal Health		
13h30-15h00	SESSION 5 SIP05	GEMINI I-III
<b>Social distancing during a pandemic</b>		
CHAIR: – Dr N. Ferguson, <i>University of London, UK</i>		
DISCUSSANTS:		
– <b>SIP05-1</b> Dr J. Edmunds, <i>Health Protection Agency, UK</i>		
– <b>SIP05-2</b> Dr M. Cetron, <i>Centers for Disease Control and Prevention, USA</i> : US community mitigation planning		
– <b>SIP05-3</b> Dr B. Schwartz, <i>National Vaccine Program Office, USA</i> : Mitigating secondary consequences of non-pharmaceutical interventions during an influenza pandemic		
15h00-15h30	COFFEE BREAK	
15h30-17h00	SESSION 6 SIP06	GEMINI I-III
<b>Equitable use of intervention strategies in a pandemic</b>		
CHAIR: – Dr K. Fukuda, <i>World Health Organisation, Switzerland</i>		
DISCUSSANTS:		
– <b>SIP06-1</b> Dr A. Nicoll, <i>ECDC, Sweden</i> : Where are we now and what needs to be done – pandemic countermeasure in Europe		
– <b>SIP06-2</b> Dr S. Jadhav, <i>Serum Institute of India, India</i> : Pandemic flu vaccine preparedness : Developing Countries Vaccine Manufacturers' perspective		
– <b>SIP06-3</b> Dr L. Hessel, <i>International Federation of Pharmaceutical Manufacturers &amp; Associations, France</i> : The industry contribution to the equitable availability of vaccines in a pandemic situation		
– <b>SIP06-4</b> Dr D. Reddy, <i>Hoffmann-La Roche, Switzerland</i> : Access to antiviral stockpiles for pandemic use – an industry perspective		
17h00-18h00	POSTER SESSION	BLUE CORNER WINTER GARDEN
18h00-19h30	SATELLITE SYMPOSIUM	FENIX I-III
– Novartis		
20h30-23h00	ESWI ANNIVERSARY DINNER	

## 17 September 2008

7h00-8h30 SATELLITE SYMPOSIUM

FENIX I-III

– Sanofi Pasteur

8h45-10h00 PLENARY 3

FENIX I-III

– **PL03-1** Keynote lecture by Dr M. Peiris, *University of Hong Kong, Hong Kong: Avian Influenza from the South East Asian Perspective*

– **PL03-2** Keynote lecture by Dr D. Swayne, *Southeast Poultry Research Laboratory, USDA/Agricultural Research Service: Veterinary influenza vaccines: Strategies and Challenges to the Development and Application of Avian Influenza Vaccines in Birds*

10h00-10h30 COFFEE BREAK

10h30-12h00 PLENARY 4

FENIX I-III

### Pandemic preparedness

#### CHAIR:

– Dr K. Fukuda, *World Health Organisation, Switzerland*

#### CO-CHAIR:

– Dr K. Ekdahl, *ECDC, Sweden*

#### DISCUSSANTS:

– **PL04-1** Dr J. Katz, *Influenza Division, US Centers for Disease Control and Prevention, Atlanta, Georgia, USA: Assessing the Pandemic Risk of Avian Influenza Viruses*

– **PL04-2** Dr N. Ferguson, *University of London, UK: Predicting the impact of layered interventions*

– **PL04-3** Dr J. Van-Tam, *University of Nottingham, UK*

– **PL04-4** Dr J. Watkins, *University of Wales, UK: Pandemic Planning at a Sub-National Level*

12h00-13h00 CLOSING SESSION

FENIX I-III

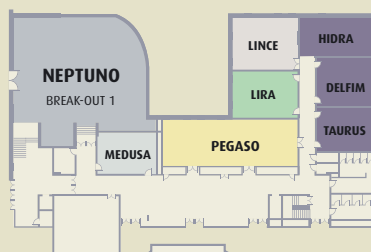
– Keynote lecture by Dr A. Osterhaus, *Erasmus MC, The Netherlands*

– Awards to young scientists

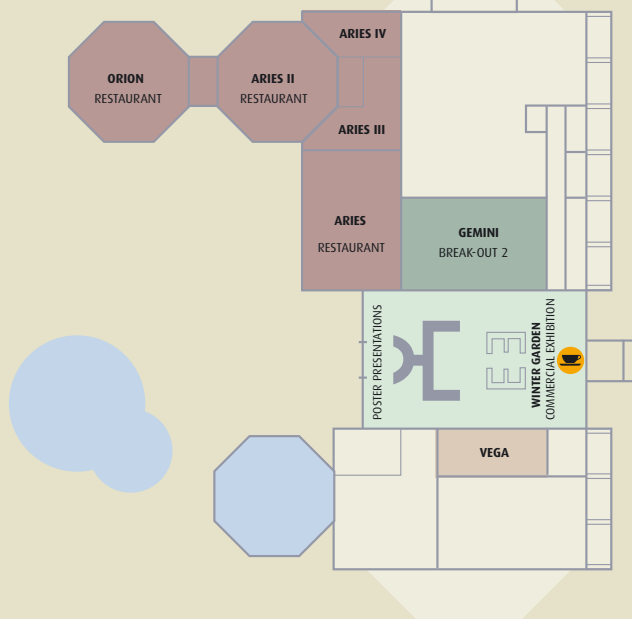
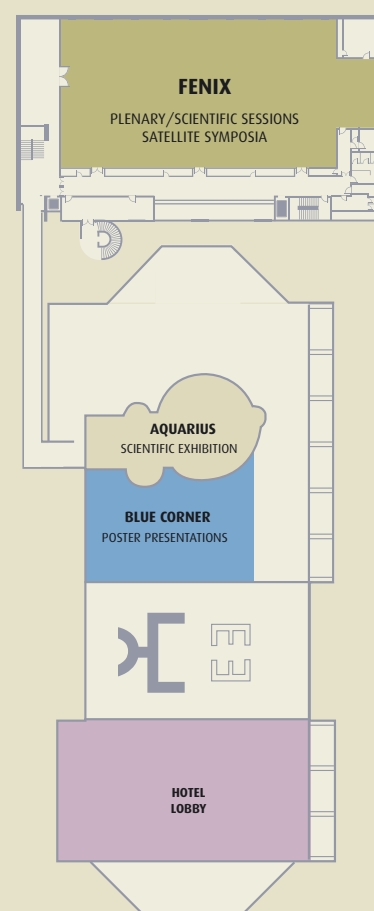
13h00-14h30 LUNCH

Plenary/Scientific Sessions	FENIX	
Satellite Symposia	FENIX	
Break-out room 1	GEMINI	
Break-out room 2	NEPTUNO	
Overflow room	NEPTUNO	
Poster Presentations	BLUE CORNER	
	WINTER GARDEN	
Press Office	VEGA	
Hospitality Suites	HIDRA/DELFIN/TAURUS	
Speaker ready room	MEDUSA	
ESWI office	PEGASO	
GCO office	PEGASO	
Registration	LOBBY	
Lunch	ORION/ARIES	
Coffee Breaks		

### LEVEL 00



### LEVEL 0





# Solvay Pharmaceuticals Influenza Research Grant

a new fund to advance scientific research in influenza

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The annual burden of influenza disease due to excess morbidity, mortality and economic implications is well documented. Accordingly, WHO and many national Health Authorities recommend the annual use of influenza vaccines especially for elderly, patient groups at risk of influenza associated complications and health care workers. Avian influenza viruses, particularly H5N1 viruses, pose a threat for a next influenza pandemic. A potential societal disaster can be best controlled by the timely use of pre-pandemic vaccines and / or pandemic vaccines. Early research on respective vaccines has demonstrated that non- adjuvanted H5N1 vaccine formulations are poorly immunogenic and require high doses of antigens for an effective dose regimen. Differences in the degree of “antigen-sparing” have been found for different adjuvanted formulations. For many adjuvants used in vaccinology, there is little information on the mode of action of these adjuvants to exert their adjuvanticity.

Based on this situation, the currently offered research grant, for the first time provided in 2009, will be granted to either new - or existing research projects of non - industrial origin, aiming at a better understanding of the mode of action of different classes of adjuvants.

## Available grant:

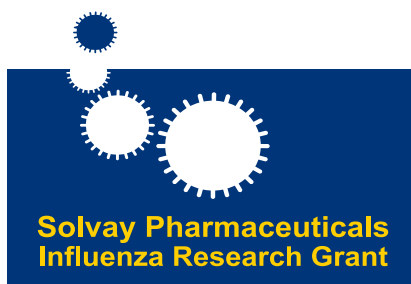
The awardees of the research grant will receive an amount of EUR 50,000 for the year 2009.

## Procedures:

Applicants should fill in a grant proposal form and are requested to provide an executive summary report of their research project. They should submit both documents to the secretariat of the Solvay Pharmaceuticals Research Grant Committee before March 31st, 2009. A Solvay Research Grant Committee chaired by an independent recognised expert in the field will evaluate all incoming proposals and nominate the winner. The winner of the grant will be notified no later than June 30, 2009. The criteria of the judgement will be predefined and made available.

## More information:

For more information, visit the Solvay booth. Or send an e-mail to [influenza@solvay.com](mailto:influenza@solvay.com), mentioning Research Grant in the subject field.



# **ORAL PRESENTATIONS**

## **Abstracts**

# PLENARY SESSIONS

SUN 14 SEPTEMBER 2008

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## OPENING OF THE CONFERENCE

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### Plagues and people: planning for pandemics

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**Dr R. Anderson**

*Imperial College of London, UK*

MON 15 SEPTEMBER 2008

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## PL01

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### PL01-1 Genes contributing to the pathogenicity of pandemic influenza viruses

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**Dr P. Palese**

*Department of Microbiology, Mount Sinai School of Medicine, New York, NY 10029*

The influenza pandemic of 1918/1919 was a unique event in recorded history, costing on the order of 50 million lives within the time span of only several months. The virus which caused this pandemic has turned out to be highly virulent in all of the systems studied, including mice, chicken embryos and macaques. At the same time, the 1918 virus is sensitive to the FDA-approved antivirals (amantadine as well as the neuraminidase inhibitors), and vaccines work perfectly well in protecting mice against a challenge with a virus containing the 1918 hemagglutinin and neuraminidase genes. Interestingly, in the 1918 virus the hemagglutinin, the neuraminidase and the PB1 genes were found to play critical roles in virulence. The hemagglutinin changed in all three pandemics of the last century (1918, 1957 and 1968) and the PB1 gene was also newly acquired in the 1957 and 1968 pandemic viruses (and possibly in the 1918 virus), suggesting that these genes carry important molecular signatures for virulence. Also, it is likely that neuraminidase was changed twice (in 1918 and 1957). For the hemagglutinins, receptor specificity and the ability to allow efficient transmission may be the crucial properties for virulence. For the PB1 gene, the PB1-F2 open reading frame, which encodes a protein with pro-apoptotic activity, may be the most important contributor to virulence. The N66S mutation in the PB1-F2, which is present in the 1918 virus, enhances virulence in the mouse model, and it is likely that this mutation in the PB1-F2 also affects virulence in humans.

The molecular dissection of the genes and their specific domains contributing to the virulence of pandemic strains will expand our knowledge of the biological and molecular properties of influenza viruses in general.

*Tumpey, T.M., Maines, T.R., Van Hoeven, N., Glaser, L., Solórzano, A., Pappas, C., Cox, N.J., Swayne, D.E., Palese, P., Katz, J.M., García-Sastre, A. (2007). A two amino acid substitution in the 1918 influenza virus hemagglutinin abolishes transmission of the pandemic virus. Science 315, 655-659.*

*Lowen, A.C., Mubareka, S. and Palese, P. (2007). Influenza virus transmission is dependent on relative humidity and temperature. PLoS Pathogens 3, 1470-76.*

*Pappas, C., Aguilar, P.V., Basler, C.F., Solórzano, A., Zeng, H., Perrone, L.A., Palese, P., García-Sastre, A., Katz, J.M., and Tumpey, T.M. (2008) Single gene reassortants identify a critical role for PB1, HA and NA in the high virulence of the 1918 pandemic influenza virus. PNAS 105, 3064-69.*

*Conenello, G.M. and Palese, P. Influenza A virus PB1-F2: A small protein with a big punch. Cell Host & Microbe 2, 207-209.*

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### PL01-2 Prepandemic vaccines: yes or no?

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**Dr A. Monto**

*University of Michigan, USA*

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TUE 16 SEPTEMBER 2008

## PL02 LECTURES BY ESWI YOUNG SCIENTISTS AWARD WINNERS



### PL02-1 The Global Circulation of Seasonal Influenza A (H3N2) Viruses

**Colin A. Russell**<sup>1</sup>; Terry C. Jones<sup>1,2,3</sup>; Ian G. Barr<sup>4</sup>; Nancy J. Cox<sup>5</sup>; Rebecca J. Garten<sup>5</sup>; Vicky Gregory<sup>6</sup>; Ian D. Gust<sup>4</sup>; Alan W. Hampson<sup>4</sup>; Alan J. Hay<sup>6</sup>; Aeron C. Hurt<sup>4</sup>; Jan C. de Jong<sup>2</sup>; Anne Kelso<sup>4</sup>; Alexander I. Klimov<sup>5</sup>; Tsutomu Kageyama<sup>7</sup>; Naomi Komadina<sup>4</sup>; Alan S. Lapedes<sup>8</sup>; Yi P. Lin<sup>6</sup>; Ana Mosterin<sup>1,3</sup>; Masatsugu Obuchi<sup>7</sup>; Takato Odagiri<sup>7</sup>; Albert D. M. E. Osterhaus<sup>2</sup>; Guus F. Rimmelzwaan<sup>2</sup>; Michael W. Shaw<sup>5</sup>; Eugene Skepner<sup>1</sup>; Klaus Stohr<sup>9</sup>; Masato Tashiro<sup>7</sup>; Ron A. M. Fouchier<sup>2</sup>; Derek J. Smith<sup>1,2</sup>

<sup>1</sup> Department of Zoology, University of Cambridge, Cambridge, UK.

<sup>2</sup> Department of Virology, Erasmus Medical Centre, Rotterdam, Netherlands.

<sup>3</sup> Universitat Pompeu Fabra, Barcelona, Spain.

<sup>4</sup> World Health Organization (WHO) Collaborating Centre for Reference and Research on Influenza, Melbourne, Australia.

<sup>5</sup> WHO Collaborating Center for Influenza, Centers for Disease Control and Prevention, Atlanta, GA, USA.

<sup>6</sup> WHO Collaborating Centre for Influenza, National Institute for Medical Research (NIMR), London, UK.

<sup>7</sup> WHO Collaborating Center for Influenza, National Institute for Infectious Diseases, Tokyo, Japan.

<sup>8</sup> Theoretical Division, Los Alamos National Laboratory, Los Alamos, NM, USA.

<sup>9</sup> Novartis Vaccines and Diagnostics, Cambridge, MA, USA.

Antigenic and genetic analysis of the hemagglutinin of ~13,000 human influenza A (H3N2) viruses from six continents during 2002–2007 revealed that there was continuous circulation in east and Southeast Asia (E-SE Asia) via a region-wide network of temporally overlapping epidemics and that epidemics in the temperate regions were seeded from this network each year. Seed strains generally first reached Oceania, North America, and Europe, and later South America. The mostly one-way nature of seeding events from E-SE Asia suggests that once A (H3N2) viruses leave E-SE Asia, they are unlikely to contribute to long-term viral evolution. If the trends observed during this period are an accurate representation of overall patterns of spread, then the antigenic characteristics of A (H3N2) viruses outside E-SE Asia may be forecast each year based on surveillance within E-SE Asia, with consequent improvements to vaccine strain selection.



### PL02-2 Influenza virus transmission: studies in the guinea pig model

**Anice C. Lowen**; Samira Mubareka; John Steel; Adolfo García-Sastre; Peter Palese

*Department of Microbiology, Mount Sinai School of Medicine, New York, NY, USA*

Human-to-human transmission is required for a given strain of influenza virus to initiate a pandemic. Furthermore, the proportion of the population affected by seasonal influenza is likely dependent on the relative efficiency with which circulating epidemic strains transmit. Nevertheless, the viral, host and environmental factors governing transmission are poorly understood. With the aim of facilitating the study of influenza virus spread, we have characterized the guinea pig as a model host for influenza. Like mice, guinea pigs are classified as small mammals and can therefore be housed in conventional rodent facilities. Unlike mice, guinea pigs are highly susceptible to infection with human influenza isolates and transmit these viruses efficiently by both aerosol and contact routes. We have observed that the seasonality of influenza seen in humans also extends to laboratory guinea pigs: transmission is reduced during the summer months. We have therefore used the guinea pig model to identify relative humidity and temperature as climatic factors which likely contribute to the seasonal periodicity of influenza. The epidemiology of influenza in the human population is also reflected in the transmission properties of human and avian influenza viruses in guinea pigs: human H3N2 isolates transmit with very high efficiency, human H1N1 isolates transmit less well, and low pathogenic avian influenza viruses do not transmit from guinea pig-to-guinea pig. These differing transmission phenotypes offer a means of mapping the viral traits that support transmission.

*This work was supported in part by the W.M. Keck and Francis Family Foundations.*

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### PL02-3 The nonstructural NS1 protein of influenza B virus interacts with nuclear speckle domains

Jana Schneider; Bianca Dauber; Thorsten Wolff

Robert-Koch Institute, Nordufer 20, 13353 Berlin, Germany

The NS1 proteins of influenza A and B viruses are essential for viral replication in normal cells since they antagonize the antiviral type I interferon (IFN) system. This is reflected by a strong attenuation of engineered mutant viruses with deleted NS1 genes (A/delNS1, B/delNS1). Interestingly, the type A delNS1 virus can replicate efficiently in IFN-deficient Vero cells, whereas the B/delNS1 virus is strongly attenuated in these hosts. This suggests that the B/NS1 protein serves another important, yet unknown function in viral replication that is distinct from IFN suppression.

We wish to define the type specific activities of the B/NS1 protein. Cell biological analyses showed that B/NS1 accumulates in the nucleus early in infection, but localizes exclusively to the cytoplasm late in infection. Hence, the trafficking of B/NS1 in and out of the nucleus appears to be regulated during infection, but little is known about the signals that control this property.

Once in the nucleus the B/NS1 protein, but not the A/NS1 protein accumulated in a dot shaped pattern. We identified these domains as nuclear speckles in which B/NS1 colocalizes with the splicing factor SC35 as shown by confocal laser scanning microscopy. Nuclear speckles are intranuclear domains that are enriched in cellular proteins involved in RNA biogenesis. The presence of B/NS1 in nuclear speckles resulted in a coalesced appearance of the otherwise irregularly shaped SC35 domains. We identified the N-terminal amino acids 1-90 of B/NS1 to be essential for nuclear speckle association and further studies are undertaken to describe a minimal motive of amino acids responsible for nuclear speckle targeting.

Since nuclear localization is a prerequisite for speckle association we hypothesized that the conserved basic amino acid clusters in the N-terminal domain are crucial for nuclear import of the B/NS1 protein. By taking a mutational approach we generated a panel of plasmids and recombinant influenza B viruses expressing NS1 proteins with alanine exchanges of two to five basic amino acids within the N-terminal domain (aa 1-93). The localization of B/NS1 mutant proteins indicated that the sequence 46-DRLHRLKRLKLE-56 is critical for nuclear targeting. By GST co-precipitation it was shown that the B/NS1 protein binds to importin  $\alpha$ . An exchange of basic amino acids within the B/NS1 sequence 46 to 56 strongly impaired importin  $\alpha$  binding. Moreover, fusion of the NS1 amino acids 46 to 56 to bacterial  $\beta$ -galactosidase mediated nuclear accumulation of the fusion protein indicating that this stretch of eleven amino acids functions as an autonomous nuclear localization signal. Thus, the basic amino acids

within the NLS not only mediated nuclear localization but were also essential for speckles association of B/NS1.

Further microscopical analyses revealed that transiently expressed B/NS1 protein also colocalizes with other speckle proteins including poly (A)-binding protein II (PABII), UAP56 and Aly/Ref. These factors are involved in the nuclear export of cellular mRNAs and some viral RNAs via the TAP/NXF1 pathway. Ongoing studies investigate a possible interaction of B/NS1 with components of the TAP/NXF1 export pathway, thereby modulating cellular or viral RNA biogenesis.



### PL02-4 Genetic analysis of cis-acting RNA sequences in influenza A

Edward Hutchinson<sup>1</sup>; Julia Gog<sup>2</sup>; Paul Digard<sup>1</sup>

<sup>1</sup> Division of Virology, Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP.

<sup>2</sup> DAMTP, Centre for Mathematical Sciences, University of Cambridge, Wilberforce Road, Cambridge CB3 0WA, UK.

The RNA of the influenza genome has a number of functions in addition to encoding proteins. In particular, it is well-established that the virus has a mechanism for selectively packaging the eight segments of its genome, and that this requires RNA sequences in the terminal parts of each segment. These functional sequences extend some way into the coding regions, and the overlap of cis-acting sequences with open-reading frames has made them difficult to study in detail. We built on a prior bioinformatics analysis to design a reverse genetics screen for cis-acting sequences in the coding region of segment 7, targeting codons with low levels of synonymous variation. The introduction of synonymous point mutations into these codons rendered the virus defective, and appears to prevent the release of virions containing a complete genome when the virus is grown in eggs. Although the mechanism for selective packaging of the genome is unknown, it is thought to involve direct interaction between the eight segments. To look for sites on other segments that interact with the sequences we identified, we allowed the defective viruses to mutate and selected for pseudorevertants. On serial passage, viruses rapidly regained wild-type phenotypes without losing the original mutations. We are now taking a forward-genetics approach to identify and characterise the interacting sites that allowed this recovery, which we hope will shed some light on the mechanism of genome packaging.

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#### PL02-5 The Sympathetic Nervous System Modulates Anti-Influenza CD8+ T cell Responses *in vivo*

**Kristie M. Grebe**; Heather D. Hickman; Kari R. Irvine; Jack R. Bennink; Jonathan W. Yewdell

*National Institute of Allergy and Infectious Diseases, National Institutes of Health, USA*

Despite the longstanding appreciation of communication between the nervous and the immune systems the nature and significance of these interactions to immunity remains enigmatic. Following influenza A virus (IAV) infection both the hypothalamic-pituitary axis and the sympathetic nervous system are activated. Using 6-hydroxydopamine (6-OHDA) treatment to chemically sympathectomize mice, we have demonstrated that SNS ablation increases the anti-IAV CD8+ T cell response approximately two fold following infection. Enhanced T cell responses in sympathectomized mice correlate with increased capacity of pAPCs to activate naïve CD8+ T cells *ex vivo*. Anti-viral CD8+ T cell responses are also enhanced by administration of a  $\beta$ -2 (but not  $\beta$ -1 or  $\alpha$ -) adrenergic antagonist. These findings demonstrate a critical role for the sympathetic nervous system in limiting CD8+ T cell responses.



#### PL02-6 The 1918 Influenza A Virus PB1-F2 Protein Contributes to the Immunopathogenesis of Viral and Secondary Bacterial Pneumonia.

**Julie McAuley**<sup>1</sup>; F. Hornung<sup>2</sup>; K. Boyd<sup>3</sup>; A. Smith<sup>4</sup>; R. McKeon<sup>1</sup>; J. Bennink<sup>2</sup>; J. Yewdell<sup>2</sup>; J. McCullers<sup>1</sup>

<sup>1</sup> *St Jude Children's Research Hospital, United States*

<sup>2</sup> *National Institute of Allergy and Infectious Diseases, United States*

<sup>3</sup> *St. Jude Children's Research Hospital, United States*

<sup>4</sup> *University of Utah, United States*

Worldwide devastation resulted from the 1918 Influenza A virus (IAV) pandemic. Distinguishing features of this virus were its ability to cause severe disease in healthy young adults and its propensity to prime for bacterial pneumonia. The interaction between viral and host immune factors to produce these outcomes is of prime interest as we prepare for a possible pandemic from the virulent H5N1 strains. One viral virulence factor of interest is a newly described pro-apoptotic protein, PB1-F2, which is cytotoxic *in vitro*. We sought to study interactions between this protein, the host and bacteria. Initially we generated a mutant IAV engineered by reverse-genetics which did not express PB1-F2, yet was otherwise isogenic to the parent virus (A/Puerto Rico/8/34). Using our influenza-bacteria synergism animal model, we demonstrated that the  $\Delta$ PB1-F2 virus had an equivalent lung viral load and was equally virulent in mice (Balb/c) when compared to the wild-type virus, but primed less efficiently for secondary bacterial pneumonia. Additionally, mice with pneumococcal pneumonia after infection with wild-type influenza had higher numbers of white blood cells present in their bronchoalveolar lavage (BAL) fluid than mice initially infected with the  $\Delta$ PB1-F2 virus. Flow cytometry used to further characterize the cellular response to infection showed an increased induction of neutrophils, T cells and monocytes within the BAL fluid of wild-type infected mice, compared to mice infected with the  $\Delta$ PB1-F2 virus. We then engineered the A/PR/8/34 IAV to express the 1918 PB1-F2 on an otherwise isogenic backbone. The IAV expressing the 1918 PB1-F2 exhibited enhanced growth characteristics *in vitro*, was more virulent in mice, induced a heightened cellular and inflammatory cytokine response, more severe pulmonary immunopathology and more efficiently primed for secondary bacterial pneumonia. This increased cellular immune response was characterized by a 1-2 log increase in pulmonary macrophages and neutrophils which was exacerbated by subsequent bacterial challenge. The priming effect of PB1-F2 on bacterial pneumonia could be recapitulated in mice by intranasal delivery of a synthetic peptide derived from the C-terminal portion of the PB1-F2 protein. Exposure of mice to this peptide, but not a peptide derived from the N-terminal region of the protein, caused increases in the cellular inflammatory response similar to those seen with the whole virus. These findings implicate PB1-F2 as an immunostimulatory protein and an important IAV virulence factor which may have contributed to the unparalleled virulence of the 1918 strain and the high incidence of fatal pneumonia during this pandemic.

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## LUNCH SESSION

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### LUSS-1 How far is it to Brussels? - Your voice in EU research funding for influenza

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**Dr C. Schmaltz**

*European Commission, Research Directorate General*

The European Commission's Research Framework Programmes (FP) represent a major funding source for influenza research in Europe. A total of 45 FP-funded influenza research project have received well over EUR 100 million FP funding since 2001, in a number of different areas from basic virology to diagnostics, vaccines, drug discovery and healthy system analysis. Based on the existing project portfolio its strengths and weaknesses will be analysed, current funding gaps identified and implications for future funding priorities will be discussed in the context of recent scientific developments and public health needs. The presentation will also explain how call topics in the FPs are drafted, stakeholders in this process will be identified and opportunities for scientific communities to make their voice heard in this process will be addressed. Scientists are encouraged to engage in a constructive and transparent dialogue with policymakers and programme managers in order to achieve maximum benefits of public research funds for European citizens and the European scientific community.

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### LUSS-2 High Pathogen Avian Influenza: EFSA's evaluation of risks in Animal Health

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**S. Dhollander; P. Have; J. Serratos**

*European Food Safety Authority (EFSA), Animal Health and Welfare (AHAW), Largo N. Palli 5/A, 43100 Parma, Italy*

EFSA is a European Institution that was created to provide independent scientific advice to the EU Commission, Parliament and Member States supporting scientific based EU legislation. EFSA's AHAW Panel reviewed the previous 4 scientific opinions on Avian Influenza (AI) (2005 2006a, 2006b, 2007a and 2007b<sup>1</sup>) on the basis of new scientific literature, surveillance data and EU outbreak observations of 2006-07 to indicate if previous probability estimates for the risk of introduction of HPAI in the EU by wild birds or import of live birds or avian products from third countries have been under- or overestimated.

In recent years wild birds are recognised to have been implicated in the geographical expansion of HPAI outbreaks, in addition to traditional transmission by infected poultry, contaminated equipment, and people. Such a role requires excretion of virus

in the absence of debilitating disease. Since the last EFSA opinion on the role of migratory birds for the spread of HPAI (2006a)<sup>2</sup>, twenty-one wild bird species have been the subject of experimental infection, and more bird species than previously thought might be involved in the spread of HPAI through shedding either in pre-symptomatic infection or in asymptomatic infection.

Given the number of cases of HPAI H5N1 in wild birds observed over the last two years in the EU, the risk of introduction and release of HPAI by wild birds appears to be occasional rather than a very rare or very frequent event. It is still unclear, however, to what extent wild bird species, from which HPAI has been isolated, act as carriers, indicators or bridge species. H5N1 virus may be circulating at an undetectable level in wild bird populations across Europe and consequently, there is a continuing risk of introduction of AI into poultry populations from infected wild birds. Pathogenic amplification of HPAI in domestic birds and subsequent spill-back to wild birds may complement the synergistic mechanism existing between domestic poultry and wild bird species responsible for the intercontinental spread of H5N1.

Importation of infected live poultry is a potential means for introduction of AI especially when the birds are in their incubation period of HPAI, infected with LPAI or of a species that does not show overt clinical signs. Hatching eggs are fumigated in the incubators, and day old chicks may have only very short post hatching virus exposure and are therefore not regarded as a big risk for HPAI introduction although HPAI infections can not be entirely excluded.

Pigeons have previously been thought to be relatively resistant to infection by the virus and therefore regarded as a low risk in the spread of HPAI H5N1. However, some recent experimental studies have shown that they are susceptible to infection developing clinical signs apparently without spreading the virus to susceptible poultry. Pigeons, therefore, pose very low threat of introducing AI viruses into poultry holdings.

Importation of fresh meat has the highest risk of introducing of HPAI with emphasis on duck meat due to the fact that the disease might not have been apparent before slaughter. Eggs for consumption may be infected with HPAI; however they do not often come into contact with poultry again. Contaminated packaging materials and trays pose a far greater risk. Egg or meat products are usually subjected to a form of heat treatment which inactivates the virus.

<sup>1</sup> EFSA (European Food Safety Authority), <sup>2</sup> <http://www.efsa.europa.eu/>.

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WED 17 SEPTEMBER 2008

## PL03

### PL03-1 Avian Influenza from the South East Asian Perspective

**Dr M. Peiris**

*University of Hong Kong, Hong Kong*

### PL03-2 Strategies and Challenges to the Development and Application of Avian Influenza Vaccines in Birds

**Dr D. Swayne**

*U.S. Department of Agriculture, Agricultural Research Service, Southeast Poultry Research Laboratory, Exotic and Emerging Avian Viral Diseases Research Unit*

Vaccines against avian influenza (AI) have had limited use in poultry until 2002 when the H5N1 high pathogenicity avian influenza (HPAI) spread from China to Hong Kong, and then multiple southeast Asian countries in 2003-2004, and to Europe in 2005 and Africa in 2006. Over the past 40 years, AI vaccines have been primarily based on field outbreak low pathogenicity (LPAI) strains that were grown in embryonating chicken eggs, chemically inactivated, emulsified in mineral oil adjuvant and injected into individual birds. Recently, recombinant viral vectored vaccines have been developed and licensed including recombinant fowlpox and avian paramyxovirus type 1 (ND) vaccines with AI H5 gene inserts. Additional vectored technologies hold promise for usage in the future possibly including baculoviruses, herpesvirus of turkeys, infectious laryngotracheitis virus, adenoviruses, attenuated influenza A viruses, AI-ND virus chimeras and bacterial vectors such as salmonella. Historically, the H5 subtype AI vaccines have demonstrated broad homosubtypic protection, primarily against H5 high pathogenicity (HP) AI viruses isolated in the early stages of outbreaks. However, as H5 viruses have become endemic and outbreaks prolonged, some drift variants with resistance to earlier H5 AI vaccines have emerged in Central America, China, Egypt and Indonesia. How widespread such drift variants are will remain unknown until more detailed genetic and antigenic analyses are conducted on field isolates. Implementation of advances in biotechnologies will overcome some existing limitations and result in vaccines that can be grown in tissue culture systems for more rapid vaccine production; provide optimized protection as the result of closer genetic relationship to field viruses through reverse genetics and gene insertions in vector systems; can be mass applied by aerosol, drinking water or in ovo administration; and provide easier strategies for identifying infected birds within vaccinated populations; i.e. DIVA.

## PL04 PANDEMIC PREPAREDNESS

### PL04-1 Assessing the Pandemic Risk of Avian Influenza Viruses

**J.M. Katz;** J.A. Belser; T.R. Maines; N. Van Hoeven; J. Achenbach; V. Veguilla; C. Pappas; K. Hancock; T. M. Tumpey

*Immunology and Pathogenesis Branch, Influenza Division, Centers for Disease Control and Prevention, Atlanta, GA, USA*

Influenza viruses with novel hemagglutinin glycoproteins sporadically emerge in humans and have the potential to result in a pandemic if the virus causes disease and spreads efficiently in a population that lacks immunity to the novel subtype. Since 1997, multiple avian influenza virus subtypes have been transmitted directly from domestic poultry to humans and have caused a spectrum of human disease, from asymptomatic to severe and fatal. To assess the pandemic risk that avian influenza viruses pose, we have used multiple strategies to better understand the capacity of avian viruses to infect, cause disease and transmit among mammals, including humans. Seroepidemiologic studies that evaluate the frequency and risk of human infection with avian influenza viruses in populations with exposure to domestic or wild birds can provide a better understanding of the pandemic potential of avian influenza subtypes. Such investigations have determined that while overall rates of human infection with avian influenza viruses appears to be low, certain types of exposure, including exposure to poultry in live bird markets, increases the risk of human infection. A second risk assessment tool, the ferret, can be used to evaluate the level of virulence and potential for host-to-host transmission of avian influenza viruses in this naturally susceptible host. Avian viruses isolated from humans exhibit a level of virulence and transmissibility in ferrets that generally reflects that seen in humans. The ferret model thus provides a means to monitor emerging avian viruses for pandemic risk and evaluate laboratory generated reassortants and mutants to better understand the molecular basis of influenza virus transmissibility. Taken together, such studies provide valuable information with which we can assess the public health risk of avian influenza viruses.

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**PL04-2 Predicting the impact of layered interventions**

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**Dr N. Ferguson***Director, MRC Centre for Outbreak Analysis and Modelling, Imperial College London, UK*

Mathematical modelling has had an influential role in pandemic planning in many countries over the last few years. Of particular note is the ability of modelling to give insight into the potential impact of combined – or layered – interventions of different types. Targeted layered interventions are now the mainstay of US community mitigation planning, and similar policies (albeit with different emphasis on pharmaceutical vs. non-pharmaceutical interventions [NPIs]) are planned in many European countries. I will start with a review of the data gaps in evaluating the likely effectiveness of NPIs, and how recent work is beginning to reduce past uncertainties. I will then discuss the limits of what NPIs alone can achieve – a relevant question for many developing countries without access to substantial stocks of vaccines or antivirals. I will then review what recent work has concluded about the likely effectiveness of layered strategies which combine NPIs, antivirals and vaccines, both for containment and mitigation.

antiviral drugs and other containment measures. Though in recent surveys, the majority of national agencies, notably in the developed world, have these plans in place, at a sub-national level, both regional and local, the picture is not quite so clear cut. There remains a gap between the ideals espoused by governmental strategies and the practical logistics of delivering care to millions afflicted with pandemic virus, in a very short time window. The practical logistics of delivering and organising care by frontline services will be vital in limiting disease impact and mortality and making the most effective use of scarce resources. The steps required for organising services and care, at a local and regional level, will be explored and some practical lessons learnt at a sub-national level in Wales, as a small region of the United Kingdom, will be presented.

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**PL04-3**

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**Dr J. Van-Tam***University of Nottingham, UK*

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**CLOSING SESSION**

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**Dr A. Osterhaus***ESWI chair, Erasmus MC*

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**PL04-4 Pandemic Planning at a Sub-National Level**

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**Dr J. Watkins***University of Wales, UK*

Attention in recent years has been concentrated on the potential threat posed by influenza A and the need for national and international collaborative efforts to limit the impact of pandemic disease. Two factors that have contributed to this heightened awareness, more than any others, have been: firstly, the continued occurrence of isolated cases and a few clusters of disease, caused by the highly pathogenic avian H5N1 subtype, in humans. For the past decade this H5N1 virus has had a devastating impact on bird populations across the world, while being implicated in disease in over 380 individuals with a 60% mortality rate, mostly in the Far East; and secondly, the recent addition of the antiviral drugs, the neuraminidase inhibitors, to our arsenal, with which we can fight this disease. While it is uncertain whether H5N1 will ever gain the potential to cause pandemic disease in humans, its very lethal nature has raised the spectre of a 1918 'Spanish Flu' scenario and caused the WHO and national governments to develop Pandemic Influenza Preparedness plans and to stockpile

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# SCIENTIFIC SESSIONS

MON 15 SEPTEMBER 2008

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## SS01 VIRUS HOST INTERACTION/ PATHOGENESIS/TRANSMISSION

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### SS01-1 Interferon antagonist functions of NS1 protein of influenza virus

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**Adolfo García-Sastre**<sup>1</sup>; Michaela U. Gack<sup>2</sup>; Randy A. Albrecht<sup>1</sup>; Satoshi Inoue<sup>3,4</sup>; Jae U. Jung<sup>2</sup>

<sup>1</sup> Department of Microbiology, Mount Sinai School of Medicine, New York, NY, USA

<sup>2</sup> Department of Molecular Microbiology and Immunology, University of Southern California, Los Angeles, CA, USA

<sup>3</sup> Department of Geriatric Medicine, Graduate School of Medicine, The University of Tokyo, Hongo, Bunkyo, Tokyo, Japan

<sup>4</sup> Research Center for Genomic Medicine, Saitama Medical School, Saitama, Japan

The influenza virus nonstructural protein 1 (NS1) functions as an inhibitor of the type I interferon response. An important consequence of NS1 expression during influenza virus infection is the inhibition of type I interferon expression. This is achieved by multiple mechanisms, including an intersection with signaling pathways leading to the transcriptional activation of the interferon promoter, and a blockade of cellular mRNA processing and trafficking. We have identified a new critical internal domain within the NS1 protein that affects type I interferon production without affecting the dsRNA binding activity of the NS1. Specific amino acid mutations in this domain resulted in attenuated recombinant influenza viruses with increased abilities to induce type I interferon production. This phenotype was due to a defect in the ability of the mutant NS1 to prevent RIG-I mediated induction of type I interferon production. We are currently investigating the mechanism of action by which these mutations affect the IFN antagonistic functions of the NS1 protein.

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### SS01-2

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**Dr Y. Kawaoka**

University of Wisconsin School of Veterinary Medicine, USA

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### SS01-3 Mutations that affect the transmissibility of influenza A viruses

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**Terrence M. Tumpey**

Immunology and Pathogenesis Branch, Influenza Division, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia, USA

The ability of pandemic strains, such as the 1918 “Spanish” influenza (H1N1) virus, to spread rapidly makes them ideal viruses for use in studying the molecular properties that confer efficient transmissibility of influenza viruses. We have developed a comparative ferret model that parallels the efficient transmission of human H1N1 viruses and the poor transmission of avian H1N1 influenza viruses in humans. The ferret model provides both contact and respiratory droplet transmission evaluation of influenza viruses. We have recently demonstrated that hemagglutinin is a major determinant of virus transmission efficiency of the H1N1 pandemic virus. In particular, receptor binding, the initial event in influenza virus infection, is essential for optimal respiratory droplet transmission of the 1918 influenza virus. The contributions of other virus genes and molecular determinants involved in efficient transmissibility of influenza viruses are currently under study and will be presented. If an avian influenza virus, such as the H5N1 subtype virus, acquires the ability to undergo efficient and sustained transmission among humans, a pandemic is inevitable. Therefore, an understanding of the molecular and biological requirements for efficient transmissibility is critical for the early identification of a potential pandemic virus and the application of optimal control measures.

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### SS01-4 Pathology of influenza in humans revisited

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**Thijs Kuiken**<sup>1</sup>; Jeffery Taubenberger<sup>2</sup>

<sup>1</sup> Department of Virology, Erasmus MC, Dr Molewaterplein 50, 3015 GE Rotterdam, the Netherlands

<sup>2</sup> Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 33 North Dr, Bethesda, MD 20892-3203, USA

Most of our knowledge about the pathology of influenza in humans is based on studies from the three pandemics of the previous century, the last of which occurred in 1968. Given the recent emergence of avian influenza H5N1 virus infection, which causes severe to fatal disease in humans associated with both respiratory and extra-respiratory complications, it is important to revisit this subject to be able to compare human and avian

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influenza virus infections and their associated lesions.

Although epithelial cells throughout the respiratory tract have specific receptors— $\alpha$ -2,6-linked sialosaccharides—and are permissive for human influenza viruses, the main lesion of uncomplicated influenza is a transient superficial tracheo-bronchitis. This corresponds with recent research showing that human influenza viruses attach predominantly to tracheal epithelial cells and progressively less to bronchial and bronchiolar epithelium.

The most important complication of human influenza virus infection is extension of infection to the lung. This results in diffuse alveolar damage, presumably due to viral infection of alveolar epithelial cells. However, the contribution of secondary bacterial infection is underestimated. Recent re-evaluation of published autopsies from the last three pandemics has shown that the majority of fatal influenza cases were associated with secondary bacterial pneumonia. This has important implications for pandemic preparedness.

Human influenza virus infection is occasionally associated with extra-respiratory complications. The sensitivity of molecular diagnostic techniques has enabled more frequent detection of virus in blood and affected tissues such as the brain, heart and muscle of such cases, suggesting the direct role of virus infection in their pathogenesis. The influenza virus has been demonstrated at the site of lesions in rare cases of influenza-associated acute encephalopathy and myocarditis, but further confirmation of the influenza virus in such extra-respiratory lesions is urgently needed. Alternatively, such extra-respiratory lesions could be caused indirectly by hypercytokinemia and other systemic effects induced by influenza-associated lesions restricted to the respiratory tract.

Human disease from avian influenza virus infections is most severe for subtype H5N1, but has also been reported for H7N2, H7N3, H7N7 and H9N2. Typically, avian influenza viruses have a preference for  $\alpha$ -2,3-linked rather than  $\alpha$ -2,6-linked sialosaccharides. In contrast to human influenza viruses, avian influenza viruses attach predominantly to alveolar and bronchiolar epithelium and progressively less to that of bronchi and trachea within the human respiratory tract. This corresponds to diffuse alveolar damage as the primary lesion of fatal H5N1 influenza, and the apparent lack of uncomplicated cases of tracheo-bronchitis. Extra-respiratory complications appear to be more common in infections with avian H5N1 virus than with human influenza viruses, and have been associated with both viremia and in situ detection of viruses in multiple extra-respiratory tissues, including the brain, liver, intestine, and lymph nodes.

There are still many gaps in our knowledge of the pathology in humans from both human and avian influenza virus infections. Comparing and contrasting these infections can only be achieved if additional influenza cases are subjected to careful pathological analysis.

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#### SS01-5 Increased pathogenesis by influenza A virus expressing a PB1-F2 protein with the N66S mutation: role of CD8<sup>+</sup> and CD4<sup>+</sup> T-cells

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Gina Conenello; P. Palese

*Mount Sinai School of Medicine, USA*

The PB1-F2 protein has been shown to be a contributing factor in pathogenesis in the mouse model. The presence of PB1-F2 from highly virulent viruses containing an N66S mutation in the amino acid sequence causes increased morbidity, mortality, lung titer and cytokine dysregulation. The mechanism of increased virulence as a result of the N66S mutation is currently unknown. In this study we focused on the contribution of the adaptive immune response and its role in the increased virulence. We chose to focus on the adaptive immune response because of the upregulation of IFN $\gamma$  and TNF $\alpha$  after day 5 during infections in wild-type mice. In addition, virus titer is increased and virus persists in the lungs an additional 2 days in mice inoculated with a virus expressing the PB1-F2 protein containing the N66S mutation. These factors lead us to hypothesize that the adaptive immune response is responsible for the increased virulence seen. CD8a C57/BL6 knockout mice were inoculated with either a wild-type virus containing a low-pathogenicity PB1-F2 or an isogenic virus that expressed a PB1-F2 protein with the N66S mutation. The mice were monitored for weight loss over a 10-day period. The lung titer and cytokine profile were measured on day 5, 7, 8 and 10 post-inoculation. Lung sections were also analyzed for infiltration of cells and general pathology. CD8a<sup>-/-</sup> mice inoculated with virus containing a PB1-F2 protein with the N66S mutation succumbed to infection by day 10. These mice showed significant lung damage as well as increased lung titer. These mice exhibited the upregulation of IFN $\gamma$  seen in the wild-type mice, but did not have a significant upregulation of TNF $\alpha$ , indicating that the TNF $\alpha$  is not essential for the increased pathogenesis. These experiments are meant to dissect the mechanism of increased virulence with N66S mutation in the PB1-F2 protein. By knocking out the CD8<sup>+</sup> T-cells we have shown that they are not essential contributors to the increased pathogenesis and that the consistent upregulation of IFN $\gamma$  may be a major cause of the increased virulence seen. We are now in the process of infecting CD4 knockout mice with a virus that expresses the PB1-F2 protein with the N66S mutation and analyzing lung titers, cytokine profiles and lung pathology.

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## SS02 CLINICAL IMPACT & DIAGNOSTICS APPROACHES

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### SS02-1 Disease burden and diagnosis of influenza in children

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**Terho Heikkinen**

*Turku University Hospital, Turku, Finland*

Numerous studies carried out in recent years have provided ample evidence of the enormous disease burden of influenza in children. During annual epidemics, the attack rates are consistently highest in children, which translates into high rates of outpatient visits in this age group. The clinical impact of influenza is not limited to viral infection of the respiratory tract, but influenza frequently gives rise to bacterial complications. Occasionally, other organ systems may also be affected by influenza virus infection. The most common complication of influenza in children is acute otitis media which develops in 40% of children younger than 3 years and 20% of children aged 3-6 years. Although the vast majority of influenza-infected children are treated as outpatients, infants and young children are frequently admitted to hospital for influenza-associated illnesses. The admission rates are highest in infants and young children among whom the rates are comparable to those in adults with high-risk conditions.

The significant impact of influenza on children calls for greater attention to be paid to effective prevention and management of this illness, especially in the youngest age groups. The neuraminidase inhibitor oseltamivir is licensed for use in children 1 year of age or older. When started early in the influenza progress, oseltamivir shortens the duration of illness and reduces the development of acute otitis media as a complication. However, the difficulty of diagnosing influenza on clinical grounds alone is an important limiting factor for the initiation of antiviral treatment in young children. Even during the peak weeks of an influenza outbreak, several other viruses circulate among children, with influenza viruses accounting for a minority of all respiratory infections. Because of substantially overlapping signs and symptoms between influenza and other respiratory viruses in young children, the clinical diagnosis of influenza remains challenging in this age group. The use of rapid diagnostic methods might be needed for optimal use of influenza antivirals in the youngest children.

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### SS02-2 Burden of influenza and other respiratory viruses in a University Hospital setting

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**Karl G. Nicholson**

*University Hospitals of Leicester NHS Trust, UK*

During the winters of 2001-02 and 2002-03, we studied 1181 children aged <71 months who were assessed or admitted to the Childrens' Hospital Leicester with acute respiratory tract illness, seizures, or an acute febrile illness. During the first season we also studied children with acute febrile gastrointestinal illness, apnoea, and other life threatening events. Age stratified assessment and admission rates/100,000 population and clinical aspects of the burden of disease for influenza will be compared with results for other respiratory viruses. During the winters 2005-06, 2006-07, and 2007-08, we studied 1252, mostly elderly, adults who were admitted to the Leicester Royal Infirmary with acute cardio-pulmonary disease and were entered into a randomised trial of the effectiveness of diagnostic tests for influenza, RSV, and pneumococcus. The goal of the study was to identify whether rapid molecular or near patient testing in comparison to standard microbiological tests affected changes in antimicrobial prescribing, patient isolation, and earlier discharge. Some provisional observations will be presented.

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**SS02-3 Human H5N1 disease: pathogenesis and treatment**

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**Menno D. de Jong**

*Oxford University Clinical Research Unit, Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam, and Department of Medical Microbiology, Academic Medical Center, University of Amsterdam, the Netherlands*

Avian influenza A (H5N1) viruses continue to cause outbreaks in birds and mammals and sporadic infections in humans. Improving insights into disease pathogenesis of human H5N1 infections and efforts to optimize clinical management are essential to prepare for the worst should these viruses cause the next influenza pandemic. H5N1 viruses cause severe disease in humans, characterized by rapidly progressive pneumonia, multi-organ dysfunction and high mortality. High levels of viral replication, propensity of the virus to cause pneumonia, dissemination to extrapulmonary organs, and an intense inflammatory response to the virus may all contribute to disease pathogenesis. The possible role of the inflammatory response suggests potential benefits for immunomodulatory treatment, but more detailed knowledge is required for a rational and safe intervention. While antiviral drugs thus remain the mainstay of treatment, the impact of oseltamivir on H5N1-associated mortality has so far been limited. Possible explanations for this include late initiation of treatment, suboptimal dosing and/or drug delivery, and development of drug resistance. The focus of treatment should be on preventing virus and immune-mediated damage by early diagnosis and effective antiviral treatment with regimens, preferably parenteral, which minimize the risk of resistance development.

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**SS02-4 Mortality benefits of statins for pneumonia when influenza is circulating**

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**Michael Rothberg<sup>1</sup>; P.S. Pekow<sup>2</sup>; C. Bigelow<sup>2</sup>; P.K. Lindenauer<sup>1</sup>**

<sup>1</sup> Tufts University, USA;

<sup>2</sup> University of Massachusetts, USA

**Background:** Pandemic flu poses one of the greatest risks to human society. Circulating strains of avian influenza have a mortality rate in excess of 50%. Although currently available antivirals might be partially effective in reducing mortality if given early and in large doses, world stockpiles of oseltamivir are insufficient to treat even a fraction of those likely to be affected. Given that most influenza mortality occurs following pneumonia, either viral or secondary bacterial treatments that prevent pneumonia mortality could theoretically lower mortality in an influenza pandemic. Evidence from several small observational studies suggests that patients with pneumonia or sepsis who take HMG-coA reductase inhibitors (statins) may have decreased mortality, but another study raised the concern that unmeasured clinical variables denoting a "healthy user" effect might account for the apparent benefit of statins. We sought to establish the

benefits of statins in a large inpatient database while adjusting for specific co-morbidities that might be associated with both statin use and mortality. We also sought to ascertain the effects of local influenza epidemics on the efficacy of statins.

**Methods:** We conducted a retrospective cohort study of patients hospitalized for pneumonia between 2002 and 2005 at 360 U.S. hospitals that participated in Premier Inc.'s Perspective, a large database used for measuring quality of care and resource utilization. In addition to the information contained in the standard hospital discharge file, Perspective includes a date-stamped log of all billed items, including medications with dose and quantity, for individual patients. Patients were included if they were >17 years old and had a principal diagnosis of pneumonia or influenza or a secondary diagnosis of pneumonia or influenza paired with a principal diagnosis of respiratory failure or sepsis. Patients whose length of stay was <2 days, who were transferred from or to another acute care facility, who did not receive either an antibiotic or antiviral medication, and those with contraindications to statin therapy (i.e. liver disease or myopathy), were excluded. Patients were considered to have received statin therapy if they received any HMG-CoA reductase inhibitor at any time during hospitalization. We developed multivariable models to estimate the effect of statins on inpatient mortality, attempting to control for confounders that might represent a "healthy user" effect. Models were adjusted for a wide range of patient (e.g. demographics, comorbidities) and hospital factors (e.g. size, teaching status). Comorbidity assessments were based on the work of Elixhauser and contained diagnoses likely to be associated with either statin use or mortality. We also adjusted for mechanical ventilation during the first 2 days of hospitalization and treatment with vancomycin as a surrogate for disease severity on admission. Finally, we analyzed the models stratified by age, to see whether the effects of statins varied across age groups. Generalized estimating equations were used to account for the effects of patient and physician clustering. To test the hypothesis that statins would be more effective for influenza-related infections, we tested for interactions between circulating influenza, as reported by the Centers for Disease Control and Prevention, and the effects of statins. Each admission was graded according to the circulating level of influenza in the local area (state) by month. Influenza activity levels included 1) none 2) sporadic, local or regional, and 3) widespread.

**Results:** Of 191,818 pneumonia patients, 160,601 (84%) met our enrolment criteria. Of those, 30,694 (19%) received a statin during the hospitalization. Rates of statin use varied by age from 5% in patients aged 18-44 years to 27% in patients aged 65-74. Compared to patients who did not receive treatment with a statin, treated patients were older (median age 74 vs. 73, respectively), more likely to be male (50% vs. 44%), white (73% vs. 68%), married (47% vs. 37%), and have health insurance (98% vs. 95%). They were less likely to smoke (11% vs. 14%), have metastatic cancer (1.4% vs. 2.6%), neurological disease (11% vs. 15%), or weight loss (3% vs. 6%) and be admitted from a nursing home (0.9% vs.

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1.7%), but more likely to have diabetes (34% vs. 19%), renal failure (10% vs. 7%), chronic pulmonary disease (52% vs. 47%), obesity (8% vs. 5.6%) and peripheral vascular disease (10% vs. 5%). They were slightly more likely to be treated with a macrolide (50% vs. 47%), had similar rates of quinolone use (61% for each), and were less likely to receive vancomycin (11% vs. 20%). Except for quinolone use, all comparisons were highly significant ( $p < 0.0001$ ). Inpatient mortality was 7.0% among non-statin users and 3.9% among statin users. After adjustment for covariates, the benefit of statins was slightly attenuated, but still associated with a decreased risk of death (OR 0.60, 95% CI 0.54-0.66). Statins were effective in all age groups, and there was no apparent interaction between age and the effect of statins. Overall mortality was 6.3% outside the influenza season and 6.8% when influenza was widespread. The adjusted odds (95% CI) of mortality among statin users compared to non-users was 0.61 (0.55-0.68) outside the influenza season, and 0.63 (0.54-0.74) when influenza was widespread.

**Limitations:** This retrospective study was conducted using administrative data only, such that unmeasured confounders could have influenced our results. However, we adjusted for a large number of co-morbidities which were strongly associated with both mortality and statin use. We were also unable to distinguish between the effects of statins begun in hospital and those given prior to admission.

**Conclusion:** For patients hospitalized with pneumonia, treatment with statins appears to decrease inpatient mortality. Adjusting for patient co-morbidities and disease severity attenuates the effect, but it remains strong. The impact is similar in or out of flu season. Randomized trials are needed to confirm these observations.

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## SS03 VIRUS STRUCTURE & REPLICATION

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### SS03-1 Multiple functions of the NS1 protein of influenza A viruses

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Robert M. Krug

*Institute for Cellular and Molecular Biology, Section of Molecular Genetics and Microbiology, University of Texas at Austin, Austin, Texas, USA*

The NS1 protein of human influenza A viruses (NS1A protein) is a small, multi-functional protein that participates in both protein-RNA and protein-protein interactions. Its N-terminal RNA-binding domain binds double-stranded RNA (dsRNA). By identifying the replication defect of a recombinant influenza A/Udorn/72 (Ud) virus that encodes a NS1A protein lacking dsRNA-binding activity, it was established that the primary role of NS1A dsRNA-binding activity is the inhibition of the interferon (IFN)-alpha/beta-induced oligo (A) synthetase/RNase L pathway and that NS1A dsRNA-binding activity has no detectable role in inhibiting the production of IFN- $\beta$  mRNA or inhibiting the activation of PKR. The rest of the NS1A protein, which is referred to as the effector domain, has binding sites for several cellular proteins, including: (i) CPSF30, a cellular factor required for the 3' end processing of cellular pre-mRNAs, thereby inhibiting the production of all cellular mRNAs, including IFN-beta mRNA and other antiviral mRNAs; (ii) p85 $\beta$ , resulting in the activation of phosphatidylinositol-3-kinase (PI3K) signalling; and (iii) PKR, resulting in the inhibition of PKR activation. The X-ray crystal structure of the NS1A effector domain in a complex with a domain of CPSF30 identifies the CPSF30 binding pocket on NS1A, which is comprised of amino acids that are highly conserved (>98%) among human influenza A viruses, and reveals that two highly conserved (>99%) NS1A amino acids outside of this pocket, phenylalanine(F)103 and methionine(M)106, participate in key hydrophobic interactions that stabilize formation of the complex. As predicted by this structure, specific single amino acid replacements in the NS1A protein of recombinant viruses result in increased production of IFN-beta mRNA in infected cells, coupled with attenuated virus replication as well as reduced virulence in mice. In infected cells the NS1A:CPSF30 complex is further stabilized by interaction with the viral polymerase complex (PB1, PB2, PA and NP). The X-ray crystal structure predicts that a hydrophilic residue at position 103 should attenuate, if not eliminate CPSF30 binding, and that any viruses that encode such a NS1A protein would be selected against during replication in humans. In fact, this is the case. Only five seasonal viruses have encoded a NS1A protein with a hydrophilic amino acid (S) at position 103, namely, three viruses isolated in 1934-1936 (including influenza A/PR/8/34 [PR8]), one virus isolated in 1954, and one virus isolated in 1976. The absence of such viruses since 1976 shows that influenza A viruses encoding NS1A proteins with S103 (PR8-like viruses) are selected against during replication in humans. This selection further demonstrates the crucial importance of NS1A protein-mediated CPSF30 binding for circulating human influenza A viruses.

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**SS03-2 Influenza virus budding**

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**Robert Lamb**; B. Chen; G.P. Leser*Northwestern University, USA*

For the influenza virus, we developed an efficient, noncytotoxic, plasmid-based virus-like particle (VLP) system to reflect authentic virus particles. The system was characterized biochemically by analyzing VLP protein composition, morphologically by electron microscopy, and functionally with a VLP infectivity assay. The VLP system was used to address the identity of the minimal set of viral proteins required for budding. In previous findings, the matrix (M1) protein was considered to be the driving force of budding since M1 was found to be released copiously into the culture medium when M1 was expressed using the vaccinia virus T7 RNA polymerase-driven overexpression system, however in our noncytotoxic VLP system, M1 was not released efficiently into the culture medium. Additionally, hemagglutinin (HA), when treated with exogenous neuraminidase (NA) or co-expressed with viral NA, could be released from cells independent of M1. Incorporation of M1 into VLPs required HA expression, although when M1 was omitted from VLPs, particles with morphology similar to wild type VLPs or viruses were observed. Furthermore, when HA and NA cytoplasmic tail mutants were included in the VLPs, M1 failed to be efficiently incorporated into VLPs consistent with a model in which the viral glycoproteins control virus budding by sorting into lipid raft microdomains and recruiting the internal viral core components. In addition, a mutational analysis of the M2 cytoplasmic tail revealed a region that plays a role in VLP assembly, possibly through an interaction with M1 and by disrupting the proper assembly of viral proteins at the site of virus budding. VLP formation also occurred independent of the function of Vps4 in the multivesicular body pathway as dominant-negative Vps4 proteins failed to inhibit influenza VLP budding.

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**SS03-3 The influenza virus RNA polymerase: a key determinant for viral host-range and pathogenicity, a target for antiviral strategies**

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**Nadia Naffakh***Unité de Génétique Moléculaire des Virus Respiratoires, URA CNRS 3015, Institut Pasteur, 25 rue du Dr Roux, 75015 Paris, France*

Genetic analyses provide evidence of a major role of influenza virus RNA polymerase in determining viral host range and pathogenicity. Analysis of the molecular mechanisms involved has been a very active field of research in the last decade. The viral polymerase has been found to interact with a number of host factors. However the significance of these interactions

with respect to viral fitness and pathogenicity remains largely unknown.

In order to examine the efficiency with which ribonucleoproteins (RNPs) derived from an avian or a human virus are assembled and interact with the cellular RNA polymerase II in the context of avian or human cells, two experimental systems were used in our laboratory. Transient reconstitution of viral RNPs was performed using viral-like reporter RNAs synthesized under the control of the human and chicken RNA polymerase I promoters, respectively. A set of recombinant influenza viruses expressing RNPs with a PB2 protein fused to a streptavidin binding peptide (Strep-tag) was produced by reverse genetics. Co-purification assays indicate that the avian-derived polymerase is assembled and interacts physically with the cellular RNA polymerase II as least as efficiently as does the human-derived polymerase, in human as well as in avian cells. Restricted growth of the avian isolate in human cells correlates with low levels of NP protein in infected cell extracts, and poor association of the NP with the polymerase as compared to what is observed for the human isolate. Overall, our data point to viral and cellular factors regulating the NP-polymerase interaction as key determinants of influenza A viruses' host-range.

Recombinant influenza viruses expressing a PB2 protein fused with the Strep-, HA- or Flag- tag are now being used for further studies of the molecular interactions between viral polymerase and host factors during the infectious cycle. A genetic approach based on two-hybrid screening in yeast has been developed in parallel. Each of the PB1, PB2 or PA polymerase subunits or PB2-PB1 or PA-PB1 complexes have been used as bait. A selection of candidate interactors is being individually validated in cell culture assays, and tested for its ability to interact with all combinations of polymerase subunit baits in yeast. Interestingly, our results suggest that co-expression of two polymerase subunits as baits can indeed allow interactions that do not occur with single subunit baits.

The viral polymerase is an appropriate target for novel antivirals that would help in developing therapy and prophylaxis of avian and human influenza infections. Only a few anti-polymerase compounds have been reported so far as being active against influenza replication in vitro or in animal models. Using the phage display method, we have selected peptides for their affinity towards purified influenza RNPs. They are being tested for their potential to inhibit the transcription/replication of a viral-like RNA in a transient expression system and for antiviral activity.

Better knowledge of the influenza virus polymerase structure and interaction with viral and host partners should lead to new antiviral approaches and drug screening strategies in the future.

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#### SS03-4 Nucleotides outside the predicted packaging sequences of the HA segment of influenza A virus influence the incorporation of this segment into virions

Elena M Carnero<sup>1</sup>; S. Lu<sup>2</sup>; A. García-Sastre<sup>3</sup>

<sup>1</sup> Mount Sinai School of Medicine, USA;

<sup>2</sup> Department of Medicine, University of Massachusetts Medical School, 364 Plantation Street, Worcester, Massachusetts 01605, USA;

<sup>3</sup> Department of Microbiology, Mount Sinai School of Medicine, 1 Gustave L. Levy Place, New York, New York 10029, USA

The influenza A virus genome consists of eight negative-sense RNA segments that must each be packaged in order to generate an infectious particle. The process by which the viral RNA is incorporated into the virion is poorly understood. Several pieces of evidence suggest that it is a specific mechanism as opposed to a random process which mediates the incorporation of the viral RNA into the virion. Specific regions have previously been described in the influenza RNA genome as necessary for the packaging of each segment. It is assumed that the packaging signals are located in different areas at both the 3' and 5' ends of each RNA segment. Specifically, in the case of the HA segment, the minimal sequences required for packaging a foreign ORF lie within the non-coding regions as well as the first nine coding nucleotides, and the last 80 coding nucleotides at the 3' and 5' ends, respectively. Based on these results, we have rescued different viruses containing the ORF of a mammalian codon-optimized version of HA flanked by the HA packaging regions. These viruses, although containing the described HA packaging sequence, are unable to efficiently incorporate the HA segment into the virion, show a reduced expression of HA in infected cells and are attenuated for growth in both cell culture and eggs. Control viruses, containing a non-codon optimized ORF flanked by the packaging regions for the segment, do not show any defects in virion incorporation of the HA segment, expression of HA protein or virus growth. HA-defective viruses were passaged in eggs at least 10 times in order to isolate possible revertant viruses. We isolated four viruses that showed an increase in the level of incorporation of HA RNA segment and a higher level of expression of HA into the virion. We sequenced the HA segment of the revertant viruses and identified different changes in each virus, most of them located in the last 80 nucleotides of the coding region of the codon-optimized HA, and one associated with a deletion of the original sequences flanking the codon-optimized HA. Viruses containing the mutations in HA that we found in the revertants were then rescued. These viruses showed the same characteristics observed with the revertant virus, thus confirming that the changes observed in the HA are responsible for their phenotypes. Our results indicate that influenza viral RNA packaging can be influenced by sequences outside the mapped packaging signals, perhaps by affecting as yet uncharacterized functional or structural domains required for RNA packaging.

#### SS04 VACCINES: CURRENT AND NOVEL APPROACHES

##### SS04-1 The TLR3 agonist, PolyI:PolyC12U, added to influenza vaccines as a nasal adjuvant induces a wide spectrum cross-protection against different subtypes including highly pathogenic H5N1 avian influenza virus

Masato Tashiro; Hideki Hasegawa; Takeshi Ichinohe

National Institute of Infectious Diseases, Tokyo, Japan

Nasal vaccines with mucosal adjuvant are candidates for future influenza vaccines. The double stranded RNA, PolyI:PolyC, is a TLR3 agonist, which is a potent inducer of innate immune and antiviral responses against homologous and heterologous influenza viruses. However clinical studies show it to be toxic in humans. Nonetheless, PolyI:PolyC12U (AMPLIGEN®) is a similar dsRNA with a mismatch in every 12 nucleotides and has a good safety profile in a Phase III clinical trial for treatment of chronic fatigue syndrome.

We have therefore used AMPLIGEN as a mucosal adjuvant added to trivalent seasonal influenza vaccines. In mice and cynomolgous monkeys inoculated intranasally with the adjuvanted seasonal vaccines, serum antibodies as well as the IgA antibody in respiratory tracts were induced against not only the homologous viruses but also hetero-subtypic viruses including different clades and subclades of avian H5N1 highly pathogenic influenza viruses. The immunized animals were also protected from lethal challenge infections with these viruses.

These results suggest that intranasal administration of seasonal influenza vaccines with AMPLIGEN provides wide spectrum mucosal immune responses against a wide range of antigenic drifted and shifted viruses including H5N1.

##### SS04-2 Universal human influenza vaccine: development, properties and phase I trial

Walter Fiers<sup>1</sup>; Marina De Filette<sup>1</sup>; Karim El Bakkouri<sup>1</sup>; Nils Lycke<sup>2</sup>; Ashley Birkett<sup>3</sup>; Xavier Saelens<sup>1</sup>

<sup>1</sup> Unit for Molecular Virology, VIB Department of Molecular Biomedical Research, Ghent University, Technologiepark 927, B-9052 Ghent, Belgium

<sup>2</sup> Department of Clinical Immunology, Göteborg University, Göteborg, Sweden

<sup>3</sup> Acambis, Inc., Cambridge, Massachusetts 02139, USA (has moved)

Matrix protein 2 (M2), the third integral membrane protein of influenza A virus, forms tetrameric proton channels. M2 is abundant on the surface of virus-infected cells. M2e, the extra-cellular part of M2-protein, contains only 23 amino acid residues which are highly conserved in all human transmissible influenza A virus strains. The canonical avian M2e sequence differs at 5 positions from humans, suggesting a tendency of

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host adaptation. M2e in the context of natural infection (i.e. as part of M2-protein) elicits only weak and transient immune responses. However, an M2e-VLP vaccine, which is efficiently expressed in *E. coli*, induces high titers of different IgG subclasses in mice and the immune response protects against lethality and morbidity after a severe influenza challenge. The vaccine consists of a Hepatitis B virus core derived VLP with 3 M2e units linked per H3c subunit. Various human influenza strains were mouse-adapted and used for challenges, e.g. H1N1, H3N2 and others. The vaccine was equally protective when intranasally administered together with a mucosal adjuvant, such as CTA1-DD. This adjuvant, derived from cholera toxin, targets only immune cells, and is completely devoid of deleterious side effects. The immune response, which is polyclonal, is long lasting and can be passively transferred; the IgG subclass responsible for protection has been identified. Pre-existing H3c antibodies do not interfere with M2e-immunogenicity. Experiments with mice deficient in IgG-receptors reveal that protection is based on ADCC (antibody dependent cell cytotoxicity) and two different IgG-receptors are involved. A tetrameric M2e-vaccine construct, mimicking native M2, induces conformational epitope-specific antibodies. Clinical grade material has been prepared for an FDA Phase I approved trial. The study involved 79 subjects and consisted of four arms, a placebo and an M2e-H3c vaccine alone or with alum or with QS21 Stimulon® as an adjuvant. Two doses were given i.m. 30 days apart. The vaccine was well tolerated, there were no safety issues, and 90% of the subjects seroconverted.

#### SS04-3 MF59™ adjuvanted influenza vaccine (Fluad®) in children: safety and immunogenicity following a second year seasonal vaccination

T. Vesikari<sup>1</sup>; A. Karvonen<sup>1</sup>; M. Pellegrini<sup>2</sup>; A. Borkowski<sup>3</sup>; N. Groth<sup>2</sup>

<sup>1</sup> University of Tampere Medical School, Tampere, Finland

<sup>2</sup> Global Clinical Research and Development, Novartis Vaccines & Diagnostics S.r.l., Siena, Italy

<sup>3</sup> Global Clinical Research and Development, Novartis Vaccines & Diagnostics GmbH & Co. KG, Marburg, Germany

**Background and Aim:** Epidemiologic observations have suggested that children have the highest attack rates of influenza during both pandemic and interpandemic periods, and that especially very young children are at substantially increased risk for influenza-related hospitalizations. Influenza vaccination is the recommended method for preventing influenza and its related complications, but the immunogenicity of conventional influenza vaccines, as well as their clinical efficacy, is considered suboptimal in young children. Improved responses can be elicited with the use of vaccine adjuvants. In a previous clinical trial in 269 unprimed healthy children aged 6 to <36 months, we showed that MF59™ adjuvanted influenza vaccine (Sub/MF59; FLUAD®, Novartis Vaccines) resulted in a greater immune response compared with a

conventional split vaccine (split; Vaxigrip®, sanofi pasteur). In this extension study, the same subjects were offered a booster dose of Sub/MF59 or split vaccine one year later.

**Methods:** Healthy children (now aged 16 to <48 months) who had been primed with two intramuscular (IM) doses of Sub/MF59 vaccine or split vaccine for the 2006/07 season, received a third IM dose of the respective vaccine (2007/08 NH vaccine formulation) approximately one year after the first dose (before the start of the 2007/08 season). Immunogenicity was evaluated by a haemagglutination inhibition (HI) assay at baseline, before the booster dose, and three weeks after. Seroconversion (SC) was defined as an HI titer of 40 or higher and seroprotection (SP) was defined as a ≥4-fold increase in HI titre from a pre-vaccination titre ≥10 or a rise from <10 to ≥40. Solicited local and systemic reactions were monitored immediately after vaccination and for the following seven days. All adverse events (AE) were recorded up to 3 weeks after injection. Six-month safety follow-up is ongoing.

**Results:** Overall, 89 children took part in the study. Both vaccines were confirmed to be safe and well tolerated after a second seasonal vaccination. Mild solicited reactions were more frequently recorded in the Sub/MF59 group, whereas AEs were more common in the split group. Baseline HI antibody titres, SP rates and SC rates were higher in the Sub/MF59 group, compared with the split group. The difference in persistence of antibody titres, approximately one year after priming, was particularly evident against the A/H3N2 strain (Sub/MF59 88% SP vs. split 40% SP,  $p<0.001$ ). For both vaccines, the immune responses after vaccination were strongest against A/H3N2, followed by A/H1N1 and B. Sub/MF59 induced significantly higher GMTs than the conventional vaccine against all three vaccine strains. All subjects in the Sub/MF59 group achieved SP against all three vaccine strains whereas split vaccine conferred seroprotection in 68% of children against the B antigen ( $p<0.001$ ). The same trend was observed for the percentage of children achieving SC, with the greatest difference between groups being for the B strain (98% in the Sub/MF59 group vs. 68% in the split group,  $p<0.001$ ).

**Conclusions:** MF59™ adjuvanted influenza vaccine was confirmed to be safe and well tolerated following a second seasonal vaccination. Baseline HI antibody titers were consistently higher in the Sub/MF59 group, confirming significantly longer persistence of immunogenicity after priming with an MF59™ adjuvanted vaccine, than with a conventional vaccine. Sub/MF59 induced higher increases in immune responses three weeks after vaccination, especially versus the B influenza strain, which is epidemiologically relevant in the pediatric population. These data further support the use of MF59™ adjuvanted vaccine as a safe and superior immunogenic influenza vaccine for young children.

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**SS04-4 Recombinant modified vaccinia virus Ankara expressing HA confers protection against homologous and heterologous H5N1 influenza virus infections in macaques**

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J.H.C. Kreijtz<sup>1</sup>; Y. Suezzer<sup>2</sup>; G. de Mutsert<sup>1</sup>; J.M.A. van den Brand<sup>1</sup>; G. van Amerongen<sup>1</sup>; B.S. Schnierle<sup>2</sup>; T. Kuiken<sup>1</sup>; R.A.M. Fouchier<sup>1</sup>; J. Löwer<sup>2</sup>; A.D.M. Osterhaus<sup>1</sup>; G. Sutter<sup>2</sup>; G.F. Rimmelzwaan<sup>1</sup>

<sup>1</sup> Erasmus MC Department of Virology, the Netherlands

<sup>2</sup> Paul Ehrlich Institut, Germany

Highly pathogenic avian influenza viruses of the H5N1 subtype have been responsible for an increasing number of infections in humans since 2003. More than 60% of humans that become infected succumb and new infections are reported frequently. In view of the pandemic threat caused by these events, the rapid availability of safe and effective vaccines is desirable. Modified Vaccinia virus Ankara (MVA) expressing the HA gene of H5N1 viruses is a promising candidate vaccine that has induced protective immunity against infection with homologous and heterologous H5N1 influenza viruses in mice. In the present study, we have evaluated a recombinant MVA vector expressing the HA of H5N1 influenza virus A/Vietnam/1194/04 (MVA-HA-VN/04) in non-human primates. Cynomolgus macaques were immunized twice and then challenged with influenza virus A/Vietnam/1194/04 (clade 1) or A/Indonesia/5/05 (clade 2.1) to assess the level of protective immunity. Immunization with MVA-HA-VN/04 induced (cross-reactive) antibodies and prevented virus replication in the upper and lower respiratory tract and the development of severe necrotizing broncho-interstitial pneumonia. Therefore MVA-HA-VN/04 is a promising vaccine candidate for the induction of protective immunity against highly pathogenic H5N1 avian influenza viruses in humans.

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**SS05 LATE BREAKERS**

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**SS05-1 Membrane-associated human airway trypsin-like protease (HAT) cleaves the influenza virus hemagglutinin at the cell surface**

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E. Boettcher; H.D. Klenk; W. Garten

*Institute of Virology, Philipps University Marburg, Germany*

The influenza virus hemagglutinin (HA) mediates both binding to cell surface receptors and fusion of viral and endosomal membranes following endocytosis in order to release the viral nucleocapsid into the target cell. HA is synthesized as a precursor protein HA0 and has to be cleaved by a host cell protease into the subunits HA1 and HA2 to gain its fusogenic potential. Therefore, cleavage of HA is essential for the infectivity of the virus and represents an important determinant of viral pathogenicity. Highly pathogenic avian influenza A viruses of subtypes H5 and H7 possess HA with a multibasic cleavage site that is processed intracellularly by subtilisin-like endoproteases furin and PC5/6. In contrast, HA of low pathogenic avian and mammalian viruses contains a monobasic cleavage site and is thought to be activated extracellularly by a secreted trypsin-like protease. However, relevant proteases in the human airway epithelium have not been identified so far. We previously demonstrated that the serine protease HAT (human airway trypsin-like protease) cleaves HA with a monobasic cleavage site and supports multicycle viral replication in vitro. In order to characterize HA cleavage by HAT in more detail and to address the question of subcellular localization of HA cleavage, we generated stable MDCK-HAT cells. Interestingly, HAT was not secreted by these cells but was expressed on the cell surface in both zymogen and catalytically active forms. We found that MDCK-HAT cells could be infected with influenza virions containing non-cleaved HA and that proteolytic processing of HA by HAT took place at the cell surface prior to endocytosis. In conclusion, our data demonstrate a novel mechanism for proteolytic activation of influenza viruses using a membrane-associated protease at the cell surface.

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## SS06 HOW TO EVALUATE VACCINE EFFECTIVENESS?

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### SS06-1 Influenza vaccination and mortality benefits: new insights, new opportunities

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Lone Simonsen<sup>1</sup>; Robert J. Taylor<sup>2</sup>; Cecile Viboud<sup>3</sup>; Mark A. Miller<sup>3</sup>; Lisa Jackson<sup>4</sup>

<sup>1</sup> George Washington University, Washington DC

<sup>2</sup> SAGE Analytica LLC, Bethesda MD

<sup>3</sup> Fogarty International Center, National Institutes of Health, Bethesda MD

<sup>4</sup> Group Health Center for Health Studies, Seattle, Washington, USA

Influenza vaccination policy in most industrialized countries has sought to reduce the mortality burden of influenza by targeting seniors  $\geq 65$  years for vaccination. However, since the 1960s some public health officials have expressed concern about the effectiveness of this strategy. Although placebo-controlled randomized trials show influenza vaccine is effective in younger age groups, few seniors over 70 years of age have been studied, even though they suffer most influenza-related deaths. Recent excess mortality studies surprisingly could not confirm a decline in influenza-related mortality in several countries even as senior vaccination coverage rose greatly. At the same time, a large number of observational studies have consistently reported that vaccination reduces the risk of dying in winter from any cause by a staggering 50%; this astonishing estimate far exceeds the assessment from excess mortality studies that less than 10% of all winter deaths are attributable to influenza. Recently, however, new studies have demonstrated substantial unadjusted selection bias in these cohort studies, which explains the substantial overestimation of vaccine benefits. We have proposed an analytical framework for demonstrating such frailty selection bias, using 5 epidemiological criteria. Without the flawed cohort studies, the remaining evidence is insufficient to indicate the magnitude of the benefits, if any, seniors derive from the vaccination program. A way forward includes a long-overdue discussion of best practices in observational studies to accurately measure vaccine benefits. Coming to a realistic view of vaccine benefits and immune senescence is an important step in itself, and clears the way for development of more effect strategies for influenza control.

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### SS06-2 Challenges in evaluating influenza vaccine effectiveness

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Kristin L. Nichol

MD, MPH, MBA. Professor of Medicine, University of Minnesota and Associate Chief of Staff for Research, Minneapolis VA Medical Center, USA

The gold standard study design for evaluating influenza vaccine efficacy and effectiveness is the randomized clinical trial. However, due to ethical constraints and other practical considerations, other study designs are often necessary for evaluating the benefits of vaccination. This is particularly true for evaluating influenza vaccine effectiveness in the elderly – a group included among the high priority groups for annual vaccination in many countries. Observational studies comprise the bulk of the studies that provide information on vaccine effectiveness in older persons, but they can be susceptible to selection bias and residual confounding. All observational studies should utilize techniques to minimize the impact of bias and confounding such as multivariable statistical analyses to adjust for measured confounders, using restriction and conducting subgroup analyses to identify groups with more homogeneous risk profiles, and taking steps to minimize information bias. In addition, other techniques such as comparison of outcomes during influenza vs. non-influenza periods and conducting sensitivity analyses can also provide additional information about the impact of potential residual bias and confounding. Even after taking into account the potential for residual bias and confounding, most studies confirm the benefits of vaccination among the elderly for reducing hospitalization and death.

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**SS06-3 Impact of influenza vaccination on mortality risk among the elderly: methodological inquiry**


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**E. Hak**

*Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht, the Netherlands, e.hak@umcutrecht.nl*

Annually, influenza epidemics are associated with increased mortality rates, notably among the elderly, though estimates of the etiological fraction vary considerably due to year-to-year fluctuation in influenza activity, co-circulation of other respiratory viruses, disparities in uptake of influenza vaccines among different populations and the methodology applied. Since most evidence of influenza vaccine effectiveness in terms of reducing mortality among the elderly has been derived from observational studies, the selection of patients for influenza vaccination may induce a confounding bias, and hence vaccine effects may be over- or underestimated. In a series of studies applying different epidemiological designs from the Netherlands, we have attempted to assess the effects of influenza vaccination taking potential sources of bias into account. First, we used national mortality statistics to determine change in the excess mortality among elderly persons during periods of increased influenza and RSV activity before and after the start of the Dutch national influenza campaign in 1995/96. After the introduction, the average annual influenza-associated mortality declined from 131 to 105 per 100,000 people (relative risk 0.80). The decline was largest in the age group 65-69 years (relative risk 0.54) and less in those aged 75 years and older, and no decline was observed in RSV-associated mortality. Second, we assessed the association between routinely unavailable, but possibly powerful potential confounders such as functional health status and uptake of influenza vaccination in 500 elderly persons. Information on these variables had no impact on the effect estimates. We concluded that the potential for confounding by such unmeasured confounders is most likely small. Third, we applied a sensitivity analysis to estimate the potential influence of an unmeasured confounder in a database study including more than 44,000 observations during 7 epidemics. After adjustment for measured confounders, influenza vaccination reduced mortality by 42% (adjusted odds ratio [OR] 0.58, 95% confidence interval [CI] 0.46 – 0.73). Only an unmeasured confounder that is present in 40% or more of the elderly, that decreases the odds of receiving the vaccination more than 70%, and that increases the odds of mortality at least threefold, would lead to a statistically insignificant, but still considerable reduction in mortality (OR 0.79, 95% CI 0.62 – 1.00). Finally, using data from the same population, we applied a variety of multivariable techniques using the associations during summer periods to quantify residual confounding bias and adjusted for such bias using different sets of confounders. Taking the ratio of influenza and summer period odds ratios, influenza vaccination was statistically significantly and inversely associated with mortality (odds ratio, 0.70 [CI, 0.52 – 0.94]) and results were similar for different sets of confounders and analytical

techniques. To conclude, the approaches applied all indicated that influenza vaccination was associated with considerable all-cause mortality reduction and that the potential for confounding bias was only small. The available evidence therefore favours annual immunization of the elderly against influenza.

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**SS06-4 Cochrane Collaboration (tbc)**


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TUE 16 SEPTEMBER 2008

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**SS07 ANTIVIRALS AND RESISTANCE**

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**SS07-1 How recently published structure of the M2 ion channel protein provides insights for developing new antivirals against this protein**

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**W. DeGrado***Department of Biochemistry and Biophysics, School of Medicine, University of Pennsylvania, USA*

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**SS07-2 The structural basis of neuraminidase inhibitor resistance**

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**A. Hay;** P. Collins; L. Haire; Y.P. Lin; J. Liu; R. Russell; P. Walker; J. Skehel; S. Martin; S. Gamblin*MRC National Institute for Medical Research, Mill Hill, London, NW7 1AA, UK*

Oseltamivir, one of the two anti-neuraminidase drugs, is currently the most widely used drug against influenza. Resistance to the drug has occurred infrequently among different viruses in response to drug treatment, including A H5N1 viruses, but most notably has emerged among recently circulating A H1N1 viruses and has spread throughout the population in the absence of drug use. Crystal structures of enzyme-drug complexes, together with enzymatic properties, of mutants of H5N1 neuraminidase have provided explanations for high level oseltamivir resistance due to the common H275Y mutation, with retention of zanamivir susceptibility, and intermediate level resistance due to the N295S mutation. The effects of recent amino acid changes in the NAs of H1N1 viruses will be considered in relation to their contribution to the fitness of viruses harbouring the H275Y mutation.

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**SS07-3 Oseltamivir resistant influenza A H1N1 viruses**

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**Maria Zambon***Virus Reference Department, HPA Centre for Infection, London, UK*

Influenza A H1N1 viruses resistant to the neuraminidase inhibitor oseltamivir emerged globally during the winter of 2007-2008. A mutation in the neuraminidase protein at position 275 (H275Y) was responsible for the antiviral resistant phenotype. Isolation of this variant did not correlate with drug treatment or transmission from a treated individual. Although H1N1 viruses with this mutation have occurred sporadically at a low frequency (<1%), such viruses were considered to have reduced replication

efficiency and transmissibility, making the widespread circulation of this emerging variant unexpected.

Our results demonstrate that 2007-2008 circulating resistant strains do not appear to have a growth disadvantage, in contrast to previous strains with this mutation, and are therefore able to compete for transmission in humans. Emergence and spread of H275Y resistance is not due to a single point source. Alterations in substrate affinity of the NA enzyme may contribute to the ability to tolerate the usually debilitating H275Y mutation, thus increasing the fitness of virus strains carrying this mutation.

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**SS07-4 Enzymatic properties of the neuraminidase of seasonal H1N1 influenza viruses provide insights for the emergence of natural resistance to oseltamivir**

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**Marie-Anne Rameix-Welti;** S. Munier; V. Enouf; F. Cuvelier; P. Jeannin; S. Legal; N. Naffakh; S. van der Werf*Institut Pasteur, France*

Surveillance of the antiviral susceptibility of influenza viruses in Europe revealed the emergence of influenza A(H1N1) viruses naturally resistant to the neuraminidase inhibitor oseltamivir (Tamiflu), with high prevalence in the population in some countries. Resistance was linked to the H275Y mutation in the N1 known to confer resistance to oseltamivir but not to zanamivir (Relenza). The aim of this work was to understand the molecular basis of the apparent fitness of the resistant H1N1 viruses that emerged during the 2007/2008 season. For a selection of H1N1 viruses isolated as part of routine surveillance, we determined the IC50 values for oseltamivir using a standard neuraminidase inhibition assay and performed kinetic analyses of sialidase activities of the neuraminidase using the MUNANA fluorogenic substrate, in the absence or presence of neuraminidase inhibitors. When compared to previously circulating H1N1 viruses, oseltamivir sensitive H1N1 viruses from the 2007/2008 season were found to have significantly reduced Km and Ki values for the substrate and anti-neuraminidase, respectively. As previously described, the N1 with the H275Y mutation exhibits about 500-fold higher oseltamivir Ki values and a two-fold increase in Km as compared to its sensitive counterparts. However, its Km for the substrate was not significantly different from that of the previously circulating H1N1 viruses. Analyses of the H1 and N1 sequences showed that sensitive and resistant viruses from the 2007/2008 season belonged to the same phylogenetic clade. A specific combination of substitutions in the N1 was found for all viruses with reduced Km values for the substrate. No specific amino acid changes

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were found in the H1 of resistant viruses which could compensate for the presence of the H275Y substitution in the N1. Mutagenesis studies are in progress to determine which specific residue in the N1 could account for the variation of the N1 enzymatic properties. Due to an increased affinity for the substrate of the NA of H1N1 viruses from the 2007/2008 season, the affinity for the substrate of the H275Y mutated N1 is comparable to that of previously circulating sensitive viruses, which may contribute to their overall fitness and ability to be transmitted. These observations underline the natural variability of NA enzymatic properties and its potential consequences in terms of antiviral sensitivity of epidemic or potentially pandemic viruses.

#### SS07-5 Multiple detection of influenza A/H3 viruses lacking the Na gene segment

O. Ferraris<sup>1</sup>; V. Moules<sup>1</sup>; C. Deleage<sup>1</sup>; S. Colin<sup>1</sup>; M. Bouscambert<sup>1</sup>; A. Hay<sup>2</sup>; M. Valette<sup>1</sup>; M. Rosa Calatrava<sup>1</sup>; B. Lina<sup>1</sup>

<sup>1</sup> CNRS FRE 3011, France

<sup>2</sup> NIMR, UK

Human influenza viruses are responsible for mild to severe respiratory tract infections. The recent focus has been on managing influenza disease because of the emerging A H5N1 which represents a potential pandemic threat. Antivirals have been seen as an important treatment option, leading to the stockpiling of neuraminidase inhibitors (NAI). Since 1999, numerous studies have been implemented to determine the rate of emergence of NAI resistant isolates. Until 2007, most of the resistant isolates have shown reduced fitness, this reduction being responsible for poor transmission rates of resistant strains. Recently, several neuraminidase framework substitutions have been shown to be responsible for reduced susceptibility to antivirals while transmission remained possible. In previous studies, out of 788 A/H3N2 viruses analysed for their NAI susceptibility, four A/H3 viruses were found devoid of fluorometric NA activity, and were characterized by the impossibility to amplify their NA segment by full length RT-PCR (from plaque-assay purified viruses) (Ferraris et al. Vaccine 2006). Morphological and phenotypical studies were carried out. Despite their NA deficient segment, the four isolates infect MDCK cells efficiently. A compensatory mechanism developed by the HA was investigated. By sequencing, we identified HA mutations associated with reduced affinity to sialic acid as confirmed by a virus binding affinity assay for sialylglycopolymers and reverse genetics assays. This is the first description of infectious influenza viruses detected from human cases that is completely lacking any NA activity. The identification of such peculiar viruses raises concerns about the possible emergence of resistant viruses, particularly in the context of a pandemic where extensive use of neuraminidase inhibitors will be proposed.

## SS08 GENETIC AND ANTIGENETIC EVOLUTION

### SS08-1 Evolution of influenza viruses and vaccine strain selection process

A. Klimov

*Influenza Division, Centers for Disease Control and Prevention, Atlanta, GA, USA*

**Introduction.** Seasonal outbreaks of influenza have a significant economic impact and cause up to 500 000 human deaths worldwide. Vaccination is the most effective tool used to control the disease. The rapid genetic and antigenic evolution of influenza A(H1N1), A(H3N2) and B viruses currently taking place in humans requires regular updates of the influenza vaccine formulation. Twice a year - in February for Northern Hemisphere countries and in September for Southern Hemisphere countries - WHO provides recommendations regarding virus variants to be used in vaccines prepared for the coming influenza season. Vaccine strain selection is a multi-sided, multi-component process, in which global influenza virus surveillance plays a crucial role. Considerations for new vaccine strain recommendations require answers to the following basic questions: Are there new antigenic variants in circulation? Are these new variants spreading? Are current vaccines able to induce antibodies to the new variants? Are any new variants useful for vaccine production? To answer these questions, the WHO Global Influenza Surveillance Network (GISN) carries out year-round monitoring of antigenic and genetic characteristics of circulating viruses, evaluates influenza activity and conducts virus isolations using different cell substrates, in addition to conducting serological evaluations of vaccinated individuals and preparations of high yield reassortants suitable for vaccine production. The recent history of seasonal influenza virus evolution has resulted in multiple examples of the complex vaccine strain selection process.

**Antigenic characterization of circulating viruses.** The hemagglutination-inhibition (HI) assay using reference post-infection ferret antisera is the WHO test of choice for virus antigenic characterization. The test allows WHO Collaborating Centers for Influenza to compare antigenic profiles of new influenza virus isolates with that of the current vaccine strain. When the HI titer of a virus tested with ferret antiserum used against the vaccine strain is markedly lower than the vaccine strain homologous titer, the tested virus can be considered as antigenically drifted. Multiyear antigenic analyses of thousands of influenza A(H3N2) viruses revealed that the average annual rate of antigenic drift is equivalent to a 4-fold difference in HI titers. The antigenic variability within an influenza season is also within the range of a 4-fold difference from the vaccine strain. These data are essential for defining new antigenic variants of seasonal influenza viruses.

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Genetic analysis (sequencing) of the hemagglutinin (HA) gene provides valuable information about different genetic groups among circulating viruses of the same type/subtype and their antigenic relationships. In particular, sequencing data allow researchers to determine specific amino acid changes in the HA molecule that may lead to the appearance of new antigenic variants. For example, all recent isolates differed from the vaccine prototype strain A/Wisconsin/67/2005 (H3N2) by three amino acids: Asp-122-Asn, His-195-Tyr, and Ile-223-Val. During the 2007-08 season, the majority of H3N2 viruses worldwide belonged to the A/Brisbane/10/2007 genetic lineage with the signature changes Gly-50-Glu and Lys-140-Ile. Several minor groups of influenza H3N2 isolates co-circulating with A/Brisbane/10-like viruses are being actively monitored by GISN.

Human serology studies. Measurement of antibodies against recent virus isolates in sera from individuals who had received previous seasonal vaccines is an important element of the vaccine strain selection process. The WHO recommendation to replace A/Solomon Islands/3/2006 (H1N1) with A/Brisbane/59/2007 in the 2008-09 vaccine formulation was largely due to the human serology data.

Vaccine candidate viruses isolated from eggs. Currently licensed influenza vaccines must be produced from viruses that have been isolated in embryonated hens' eggs. However, the vast majority of laboratories worldwide use Madin-Darby Canine Kidney (MDCK) cells for virus isolation. In recent years, the responsibility of virus isolation in eggs has fallen on the WHO Collaborating Centers for Influenza. The recent evolution of human influenza viruses, in particular of the H3N2 subtype, has led to a decreased ability to infect chicken embryos. Very few 2007-08 H3N2 viruses have been isolated in eggs, thus dramatically narrowing the spectrum of available vaccine candidates. In addition, growing viruses in eggs can cause changes in patterns of glycosylation of the HA molecule. Hemagglutinins of recent influenza B viruses, for example, lose the 197-199 glycosylation site during passages in eggs.

High-yield vaccine reassortants. To allow the necessary level of vaccine production, reassortants between the recommended influenza A vaccine strains and a donor strain of high yield in eggs, the A/PR/8/34 (H1N1) virus, are made. During the reassortment and cloning, additional mutations affecting the antigenic profile of the HA molecule can occur. For example, all reassortants between the WHO recommended A/Moscow/10/99 (H3N2) and A/PR/8/34 prepared in 2000 by 4 different laboratories had amino acid changes in HA and were antigenically different from the wild type virus. A/Moscow/10/99 had to be replaced by A/Panama/2007/99.

Time constraints seriously affect vaccine strain selection. Due to the fact that vaccine production and quality control, and vaccine distribution and immunization campaigns require at least 8

months advance preparation, vaccine strain recommendations must be made during the preceding influenza season, sometimes even before the season has peaked, in an environment where the information about circulating epidemic strains is often rather limited.

Conclusions. GISN and the WHO Collaborating Centers conduct year-round influenza virus surveillance focused on vaccine strain selection. Timely exchange of information regarding antigenic and genetic characterization of circulating viruses as well as the sharing of viruses within GISN, particularly viruses isolated from eggs, is crucial for vaccine strain selection. New approaches such as cell grown and reverse genetics derived vaccines are needed for the future development of the best possible influenza vaccines.

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#### SS08-2 Mapping the molecular determinants of the antigenic evolution of the influenza A (H3N2) virus

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**Björn Koel;** Theo Bestebroer; Chantal Baas; Gaby Vervaet; Emmie de Wit; Monique Spronken; Albert D.M.E Osterhaus; Derek J. Smith; Ron A.M. Fouchier

Annual influenza A virus epidemics affect approximately 5-15% of the world's population and are responsible for an estimated 0.25 – 0.5 million deaths annually worldwide. The principal way of combating this problem is vaccination. Antibodies against the haemagglutinin (HA) protein provide protective immunity against infection and therefore HA is the main component of influenza vaccines. However, antigenic drift - the accumulation of mutations in HA as a result of population immunity - warrants the need for frequent updates of the vaccine. By means of the haemagglutination inhibition (HI) assay, differences in the antigenic phenotype can be identified, so this assay is widely used for detection of antigenic drift variants. Since the introduction of the H3N2 subtype in the human population in 1968, at least 11 "antigenic clusters" of viruses have emerged, each of which has been subsequently replaced by antigenically distinct viruses (Smith et al., Science 2004). We selected representative prototype viruses for each of the antigenic clusters, which enabled us to study the molecular determinants of the antigenic evolution of the H3N2 subtype viruses in detail using reverse genetics techniques.

By constructing HA chimeric influenza viruses of the H3N2 subtype and analysis of the HI data by antigenic cartography methods, we have determined the contribution of different regions of the HA molecule to the antigenic phenotype of H3N2 subtype viruses. The HA1 domain was solely responsible for the antigenic phenotype. Within HA1, the residues around the receptor-binding site primarily dictated the antigenic phenotype of the influenza

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A (H3N2) virus. Through site directed mutagenesis experiments and using antigenic cartography methods, we have identified all amino acid changes that have been responsible for transitions in antigenic phenotypes observed since 1968. Of all the cluster transition mutations, only one to three amino acid substitutions had a significant antigenic effect, whereas the remainder of the mutations were antigenically neutral. We have thus mapped the molecular determinants of the antigenic evolution of influenza A (H3N2) viruses in humans over a 35-year period, increasing our understanding of influenza A virus evolution, and potentially aiding in future vaccine strain selection efforts.

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### SS08-3 The antigenic and genetic evolution of equine influenza virus (H3N8) from 1976 to 2007

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**N.S. Lewis**<sup>1,3</sup>; J.M. Daly<sup>1</sup>; C.A. Russell<sup>2</sup>; D. Horton<sup>3</sup>; J.A. Mumford<sup>3</sup>; J.L.N. Wood<sup>3</sup>; D. Elton<sup>1</sup>; D.J. Smith<sup>2,4</sup>

<sup>1</sup> Animal Health Trust, Lanwades Park, Kentford, Newmarket, Suffolk, CB8 7UU, UK

<sup>2</sup> Department of Zoology, University of Cambridge, Downing Street, Cambridge, CB2 3EJ, UK

<sup>3</sup> Cambridge Infectious Disease Consortium, Department of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge, CB3 0ES, UK

<sup>4</sup> Department of Virology, Erasmus Medical Centre, Rotterdam, the Netherlands

Influenza virus exhibits phenotypic variation over time when infecting some host species. The primary component of this results from variation in the major surface glycoprotein, haemagglutinin (HA), which causes an antigenic difference, and allows the virus to escape prior immunity in the host population.

Equine influenza (H3N8) virus is a major respiratory pathogen in horses, with equine influenza outbreaks resulting in significant economic loss to equestrian industries.

Vaccination with HA is the main method used to control or prevent influenza infection. It is important that the vaccine strain is representative of the currently circulating virus strains to adequately protect horses against clinical signs of influenza and prevent sub-clinical virus shedding.

The haemagglutinin inhibition (HI) assay, typically carried out using ferret antisera, measures phenotypic change in the influenza virus. Here, we compile HI assay data for eighty (H3N8) virus evolutions spanning the period 1976–2007. However, the HI assay is considered to be of low resolution. We apply a new method called antigenic cartography to equine influenza viruses, having already been applied to human influenza viruses, to allow better resolution of antigenic differences.

We use antigenic cartography to distill the HI assay tabular data into an antigenic map: a visual representation of the inter-relationship between the different strains and the evolution of equine influenza (H3N8) viruses over time. We examine the spatial relationships between evolving strains and vaccine strains in use prior to the application of antigenic cartography, and identify strains where we would expect immune escape

from vaccine protection but where analysis by classical reading of HI tables did not find sufficient antigenic drift.

To better understand the genetic basis for antigenic evolution, we study aligned HA1 domain sequence data phylogenetically and use antigenic cartography to directly compare the genetic evolution with the phenotypic evolution of the HA. We identify one or two amino acid changes which may cause significant changes in HI reactivity. We identify candidate amino acid substitutions potentially contributing to outbreaks of equine influenza or antigenically defining a particular lineage that could be key in the evolutionary dynamics.

Antigenic cartography has increased the value of equine influenza surveillance data and was formally adopted at the International Meeting of OIE Experts on Control of Equine Influenza for antigenic characterisation of HI data for equine influenza vaccine strain selection into the future.

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### SS08-4 Reassortment and recombination in influenza

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**Jonathan Bollback**<sup>1</sup>; J. Yu<sup>2</sup>; R. Nielsen<sup>2</sup>; A. Rambaut<sup>1</sup>; A. Leigh-Brown<sup>1</sup>

<sup>1</sup> University of Edinburgh, UK

<sup>2</sup> University of Copenhagen, Denmark

Influenza poses a significant threat to human health, and periodically, new virulent strains evolve that result in high death tolls among humans. A common feature of influenza evolution involves the reassortment of genomic segments among strains. This process can act to combine beneficial mutations residing on different segments, potentially giving rise to new virulent strains. Reassortment has been implicated in all of the severe epidemics in the past and will likely continue to play a major role in future epidemics. In addition, reassortment has been implicated in the shifts between different host lineages (e.g., swine and humans). While the importance of reassortment in influenza virulence is well known, evolutionary rates and patterns of reassortment are little understood. A better understanding of these evolutionary processes provides the basis for predicting the evolution of novel strains and understanding the evolutionary constraints that can be leveraged in the development of vaccines and anti-virals.

To better characterize reassortment in influenza, we analyzed data from available whole genome sequences of avian A strains H9N2 and H5N1, and human A strains of H3N2 from New York collected over a 12-year period. Using a stochastic model of reassortment, we estimated rates of reassortment among segments. We find reassortment among all genomic segments in both the avian and human lineages, though the patterns of reassortment were qualitatively different between the avian H9N2 and H5N1. Both H9N2 and H5N1 showed significantly lower relative rates of reassortment among the segments encoding

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the polymerase components (i.e. the acidic polymerase subunit (PA), the basic polymerase subunit one (PB1), and the basic polymerase subunit two (PB2); Figure 1). Preliminary analysis of the human H3N2 data reveals a similar pattern. These patterns suggest that epistatic interactions are important in restricting the reassortment among the polymerase components.

A second evolutionary force that has been generally discounted is intra-genic recombination. While a handful of studies have found evidence for intra-genic recombination, these have been subsequently shown to be artefacts of the analysis or artefacts of data collection rather than genuine signatures of recombination. We have analyzed the avian H9N2 and H5N1 whole genome data sets for the signature of intra-genic recombination. By appropriately modelling the evolution of influenza, we have avoided biases that are likely to give rise to false signatures of recombination. For example, our analysis accommodates factors such as rate heterogeneity and error in phylogenetic estimation. We find evidence for intra-genic recombination in both avian serotypes with the strongest signatures occurring in the coding regions of PB1 and PB2 (Figure 2). Interestingly, we fail to detect recombination within the segment encoding the haemagglutinin protein (HA). While we can not entirely exclude the possibility that the observed recombination is a laboratory artefact, the patterns of exchange observed on the phylogeny are not consistent with laboratory recombination.

Figure 1. Relative rates of reassortment in avian H5N1. Reassortment among the polymerase components are shown in grey.

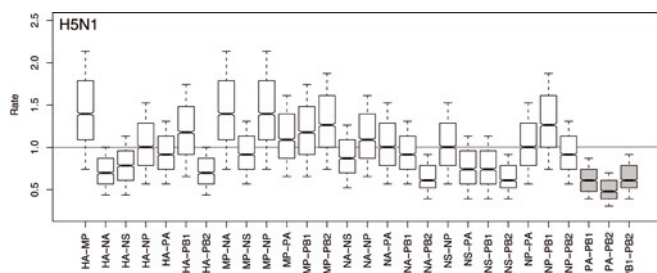
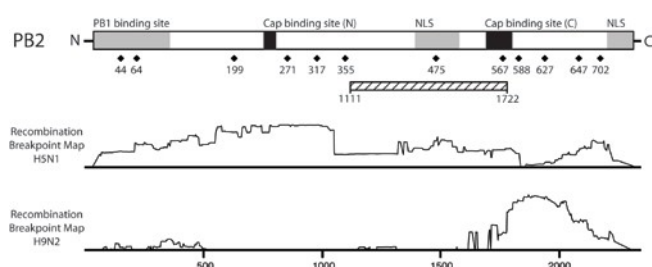


Figure 2. Genetic map of avian A influenza segment 1. The segment encodes the PB2 (basic polymerase subunit 2) protein. Functional regions of PB2 are shown as grey and black boxes (NLS, nuclear localization signal). N represents the N-terminus of the protein while C represents the C-terminus. Host determining sites are shown as diamonds and labelled by the amino acid residue – some of these sites have been shown experimentally to confer growth of avian influenza in mammalian cells (198, 317, 355, 627, 647, and 702). The cross-hatched horizontal bar represents a region (labelled by nucleotide position) that has been experimentally identified as a locus conferring the ability of avian A influenza to replicate on mammalian host cells. The traces at the bottom represent a map of support — LRT statistic values that are greater than zero.



## SS08-5 Influenza A packaging control by RNA/RNA interactions: identification of critical domains also controlling genetic reassortment

E. Fournier<sup>1</sup>; V. Moules<sup>1</sup>; B. Essere<sup>1</sup>; M. Bouscambert-Duchamp<sup>1</sup>; S. Ludwig<sup>1</sup>; M. Yver<sup>1</sup>; M. Ottman<sup>1</sup>; M. Rosa<sup>1</sup>; D. Thomas<sup>2</sup>; R. Marquet<sup>3</sup>; B. Lina<sup>1</sup>

<sup>1</sup> CNRS FRE 3011 Lyon, France

<sup>2</sup> CNRS UMR 6026 Rennes, France

<sup>3</sup> CNRS UPR 9002 Strasbourg, France

During the viral cycle, one copy of each vRNP is encapsidated, leading to the release of infectious viral particles. Terminal non-coding and coding regions of viral genomes have been shown to be crucial for segment packaging. Several studies suggest that vRNPs are selected for incorporation into the viral particle through RNA/RNA interactions, which are supposed to be located in packaging regions. However, there is no clue offered as to the precise molecular mechanism that governs this selection. Nonetheless, it has been demonstrated that genetic reassortment can occur during coinfection of a single cell by 2 different viruses of the same or of different subtypes, the latter allowing the emergence of new subtypes (i.e. A H1N2 or pandemic viruses). The mechanisms controlling the emergence of these reassortant viruses have not yet been elucidated. However, several preliminary studies suggest a link between the mechanisms of gene segment packaging and genetic reassortment.

In order to analyse RNA/RNA interactions, we have produced vRNAs from avian or human influenza A viruses of different subtypes in vitro which have been associated with purified NP protein. By using band shift experiments with these reconstructed vRNA/NPs, we have demonstrated the existence of specific RNA/RNA interactions between vRNAs. Subsequently, we have identified short domains of interaction in the vRNAs by chemical probing. These domains are located in known packaging regions, the non-coding and coding terminal regions of the gene segments. Their importance for packaging has been subsequently checked by mutagenesis by using reverse genetic experiments, confirming an altered packaging in mutated viruses. The interactions detected between the different vRNAs/NP suggest the existence of a macromolecular structure involving the different vRNAs. According to this result, we propose a putative model of vRNP organization in the viral particle. This model, proposed from the interactions observed in vitro with the vRNAs/NP, is consistent with the results obtained by tomomicro-EM experiments.

Natural genetic reassortment between influenza viruses of different subtypes is extremely rare. The vRNP interactions required to build the macromolecular complex may be a critical point for the control of genetic reassortment. Using in vitro coinfection mixing human and LPAV or HPAV in BSL-3-4 facilities,

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we observed that the rate of genetic reassortment is very low. Even with a reverse genetic system implying a competition between avian and human HA, NA and PB1 gene segments, the number of reassortant viruses detected is very low, suggesting a tight control of genome assembly.

By mutagenesis of the specific packaging regions, we have transformed the avian HA and NA packaging regions into human ones. Reverse genetic experiments using these mutated HA and NA gene segments provoked an increase in HA or NA segment incorporation, showing a clear functional link between the domains involved in vRNP packaging and those controlling genetic reassortment.

This study confirms the existence of RNA/RNA interactions between vRNP in influenza A viruses. In addition to this, we propose a network of interactions between the different gene segments in a viral particle, and put forward that there can be a functional link between the regions involved in the packaging and those controlling genetic reassortment mediated by RNA/RNA interactions.

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## SS09 ANIMAL FLU-ECOLOGY

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### SS09-1 Avian influenza in the live bird markets in the U.S.

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David Suarez<sup>1</sup>; Mia Kim<sup>1</sup>; Jan Pedersen<sup>2</sup>; Dennis Senne<sup>2</sup>

<sup>1</sup> Southeast Poultry Research Laboratory, Athens, GA, USA

<sup>2</sup> National Veterinary Services Laboratories, Ames, IA, USA

Surveillance of the live bird markets in North-eastern U.S. has routinely demonstrated the presence of low pathogenic avian influenza virus. On at least three occasions, specific lineages of virus have persisted in the market system including a H5N2 lineage related to the Pennsylvania 83 HPAI outbreak in 1986-1989, a different H5N2 lineage in 1993, and an H7N2 lineage from 1994-2006. Active control programs were able to eliminate these circulating viral lineages, but viruses of many different subtypes are routinely reintroduced back into the market system, although seldom are related viruses isolated on more than one occasion. The live bird markets present a niche of opportunity for the virus, presumably because of the congregation of multiple different species from many different sources in a confined area. The role of ducks and other waterfowl have been implicated as sources of infection in many different studies. The isolation pattern suggests that when a well established lineage is in the market, such as H7N2, other subtypes are isolated at a much lower frequency, presumably from direct competition. When the H7N2 lineage was finally eradicated in early 2006, the increase in isolation of other influenza subtypes was observed, specifically H5N2 LPAI viruses. Over 35 H5N2 viruses from the LBMs were sequenced and analyzed to determine the pattern of infection. In addition, a sequence from a number of H5 wild bird isolates from the Eastern U.S. were included for analysis to determine if similar viruses were circulating in wild birds during the same period of time. Preliminary analysis shows that multiple H5N2 viruses were introduced into the market, but only two distinct lineages of virus were isolated. Seven genetically similar viruses were seen in May-July of 2006, but were not isolated again. A second lineage of at least 20 viruses was first seen in October 2006 and was the predominant isolate until the end of the study in April 2007. The Northeast LBM system is probably the largest in the U.S. and has consistently been a niche for flu where other markets have not been. However, the current control measures, including quarterly market closures and testing of parent flocks, used in the live bird markets appear inadequate for preventing the introduction of new viruses, therefore new approaches are needed to control the problem.

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**SS09-2 The role of migratory birds in the origin and perpetuation of influenza viruses**

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**Robert G. Webster**

*PhD, FRS. Department of Infectious Diseases/Division of Virology  
St. Jude Children's Research Hospital  
332 North Lauderdale, Memphis, TN 38105-2794, USA*

Migratory waterfowl of the world are the natural reservoirs of influenza viruses of all known subtypes. It is unknown whether these waterfowl perpetuate highly pathogenic (HP) H5 and H7 influenza viruses. Longitudinal surveillance studies in migratory waterfowl for over 25 years at two sites in North America (Delaware Bay, New Jersey and Alberta, Canada) have established that 14 of the 16 subtypes of influenza viruses are perpetuated in the waterfowl of the Americas. To date, H14 and H15 have not been found. In contrast, all 16 subtypes have been isolated in Europe. In this report, attention will be given to the phenomenon of surveillance 'hot spots' where influenza viruses have been isolated from shorebirds with relatively high frequency (10-15%), whereas shorebirds examined in Alaska or Europe have very low or undetectable levels of influenza viruses. Over six million waterfowl of 35 species migrate between Eurasia and Alaska, yet to date there is no evidence for the Asian HP H5N1 in the Americas. Another concept to be discussed is the shedding of virus in migratory waterfowl by the respiratory versus the intestinal (fecal) spread. There is increasing evidence for influenza viruses to be shed by either route; in particular the Asian HP H5N1 viruses are shed for longer periods by the respiratory route.

While much attention has been given to the role of migratory waterfowl in the spread and perpetuation of Asian HP H5N1 viruses, less attention has been given to the emergence of other subtypes including the H7 and H9 influenza viruses. In this presentation, we will address the emergence of highly pathogenic H7N3 influenza viruses in North America and the spread of H9N2 influenza viruses in Eurasia.

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**SS09-3 Avian influenza virus; virus and bird ecology**

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**Vincent Munster; A.D.M. Osterhaus; R.A.M. Fouchier**

*Department of Virology, Erasmus MC, the Netherlands*

The recent introductions of highly pathogenic avian influenza (HPAI) H5N1 virus in wild birds and its subsequent spread throughout Asia, the Middle East, Africa and Europe has put a focus on the role of wild birds in the geographical spread of HPAI H5N1. Large-scale surveillance programs are ongoing to determine the potential role of wild birds in H5N1 virus spread and to serve as a sentinel system for introductions into new

geographical regions. Importantly, this increased wild bird surveillance also yields new data on low pathogenic avian influenza A (LPAI) viruses. Although various new host species for LPAI viruses continue to be identified, our understanding of the ecology of LPAI viruses has remained unchanged. Migratory ducks are generally regarded as the main reservoir for most LPAI viruses, and are thus most frequently targeted in surveillance studies. Other reservoir species such as resident birds and migratory geese, swans, waders and gulls are studied less frequently. Here we report new data that shed new light on the epidemiology and ecology of LPAI in several reservoir species: Virological and serological assays using samples from thousands of shorebirds failed to detect significant LPAI virus prevalence in several wader populations, except those in Delaware Bay, USA. Thus, European waders do not appear to play a significant role in the epidemiology of LPAI virus, in contrast to what has been suggested for North America. In 2005, the 16th HA subtype of LPAI was described, and was detected in gull influenza viruses. Using historical and novel strain collections, we obtained numerous H13 and H16 subtype viruses from gulls and terns over a 30-year period. Full genome sequences were analyzed to study the long-term evolution of these viruses. The collection of cloacal and oropharyngeal samples from several different bird species allowed an assessment of the role of the respiratory tract as a site of replication for LPAI viruses. Although LPAI viruses are thought to be transmitted primarily via a fecal-oral route, infection of the respiratory tract may suggest alternative routes of virus transmission in some wild bird species. LPAI viruses are generally believed to be apathogenic for wild birds. We analyzed wild swans equipped with satellite transmitters to determine the effect of LPAI infection on the performance of Bewick's swans. Infected swans displayed delayed migration, travelled shorter distances and fed at reduced rates. This suggests that infection with LPAI viruses may have a higher clinical impact on wild birds than previously recognized. Studies on LPAI-infected migratory ducks also suggest that wild birds are suffering more from infection than realized previously. In addition to surveillance of migratory birds in rural wetlands, we conducted surveillance studies in urbanized areas. Resident waterfowl in parks and ponds in highly urbanized areas in the Netherlands were tested for the presence of LPAI viruses. Influenza A viruses and antibodies against influenza A virus were detected in resident waterfowl. These results imply that in case of widespread HPAI H5N1 virus prevalence in wild birds, control measures should not be limited to rural areas, but should also be considered for highly urbanized areas.

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#### SS09-4 Multiple incursions of H5N1 highly pathogenic avian influenza virus into Europe

Ian H Brown; B.Z. Londt; B. Choudhury; J. Banks

*Veterinary Laboratory Agency, UK*

Since late 2005 following a westward spread, the first cases of H5N1 HPAI were reported in poultry in Turkey. This was the forerunner of a series of incursions either through poultry movement (or associated products) or via migratory wild birds. Due to enhanced surveillance in wild bird populations in the European Union during 2006, a number of incursions were shown to be present in several regions across Europe in wild birds in the absence of infection in poultry. In some cases there was spill-over of such infection into the poultry population, but apart from one outbreak in Hungary in June of that year there was very limited spread. There was considerable heterogeneity in these viruses consistent with maintenance in small eco-systems and multiple transmissions between wild bird populations. In the summer of 2007 after a winter period of H5N1-free detections in wild bird populations there were further incursions with spill-over into poultry populations (mainly backyard but also some major outbreaks in commercial units). Viruses associated with poultry in the early part of 2007 showed a close causal relationship with the viruses circulating in 2006, however the viruses that appeared later in the year appeared to be a new and independent introduction. Viruses closely resembled those present in poultry populations in the Middle East circulating in the early part of 2007. Further incidents in wild birds in 2008 in the UK provided a unique opportunity to study virus epidemiology in a local wild bird population. The data will be presented and discussed.

#### SS09-5 Immunity to H1N1 swine influenza virus can partially protect pigs against a low pathogenic H5N1 avian influenza virus

D. Braeckmans<sup>1</sup>; E. Cox<sup>2</sup>; A. De Vleeschauwer<sup>1</sup>; K. Van Reeth<sup>3</sup>

<sup>1</sup> Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University, Belgium

<sup>2</sup> Laboratory of Virology and Immunology, Faculty of Veterinary Medicine, Ghent University, Belgium; <sup>3</sup>Ghent University, Belgium

**Introduction:** There is concern that the highly pathogenic (HP) H5N1 avian influenza (AI) virus may spark the next human influenza pandemic if the virus acquires the ability to spread efficiently between humans. The lack of immunity to the H5 haemagglutinin (HA) would make the human population highly vulnerable. However, most adult humans have previously experienced infections with H1N1 and/or H3N2 influenza viruses and the human H1N1 virus shares the same N1 subtype with H5N1. Antibodies to the N1 of a contemporary human H1N1 virus

were shown to cross-react with human H5N1 isolates [1]. In addition, vaccination with a DNA vaccine expressing the N1 of this human virus partly protected mice against a lethal H5N1 virus challenge. Partial protection against H5N1 challenge has even been demonstrated in mice with infection immunity to H9N2 [2], and this appeared to be due to cross-reactive cell-mediated immunity. These data have prompted us to study the extent of cross-protection between H1N1 and H5N1 influenza viruses in the pig model of influenza. The challenge virus used was a low pathogenic (LP) H5N1 AI virus. This virus shows structural similarities with HP H5N1 viruses but requires less stringent biosafety measures and it was shown to replicate relatively well in the porcine respiratory tract in preliminary investigations.

**Materials and methods:** Twenty-six conventional influenza virus-seronegative pigs were used. At the age of 5 weeks, 13 pigs were inoculated intranasally with 7.0 log<sub>10</sub> EID<sub>50</sub> of the H1N1 swine influenza virus sw/Belgium/1/98 (H1N1-H5N1 group) and 13 pigs were left uninoculated (H5N1 challenge control group). Four weeks later, all pigs were challenged intranasally (7.0 log<sub>10</sub> EID<sub>50</sub>) and intratracheally (7.5 log<sub>10</sub> EID<sub>50</sub>) with the LP H5N1 AI virus duck/Minnesota/1525/81. Between 1 and 5 days post challenge (dpc) two pigs per group were euthanized daily. Samples of the entire respiratory tract were collected and used for virus titration in MDCK cells. All pigs were monitored clinically from 5 days before until 8 dpc with H5N1 or until euthanasia. Serum was collected from all pigs at the time of the H5N1 challenge and from the remaining pigs (3 per group) 14 dpc. The sera were examined in haemagglutination-inhibition (HI), virus-neutralisation (VN) and neuraminidase-inhibition (NI) tests against the H1N1 and H5N1 viruses used. Peripheral blood mononuclear cells (PBMCs) were collected from 6 pigs per group at the time of the H5N1 challenge and from the remaining pigs 3 weeks pc with H5N1. The cells were restimulated in vitro with the H1N1 and H5N1 viruses mentioned or with a LP H5N2 AI virus (chicken/Belgium/150/99). Lymphocyte proliferation was examined by uptake of [<sup>3</sup>H] thymidine and IFN-gamma secretion was determined by a commercial ELISA.

**Results:** The H5N1 challenge produced mild to moderate clinical signs including fever, depression, loss of appetite, tachypnoea, laboured abdominal breathing and coughing in all challenge control pigs, while the pigs of the H1N1-H5N1 group showed complete clinical protection. The H5N1 virus was isolated from the lungs of all challenge control pigs, from the trachea of most pigs and from the nasal mucosa, nasopharynx and tonsils of some pigs (see Table 1). In the H1N1-H5N1 group, in contrast, H5N1 virus was isolated from the lungs of only 2/10 pigs and from the nasal mucosa of 3/10 pigs, while all other samples tested virus-

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negative. At the time of the H5N1 challenge, the control pigs were negative in all serological tests. The pigs of the H1N1-H5N1 group, which had been inoculated with H1N1 one month earlier, had HI and VN antibodies to H1N1, but not to H5N1 (Table 1). These pigs also had NI antibodies to H1N1, which cross-reacted with H5N1. The H1N1-immune pigs showed a 19-fold higher mean lymphoproliferative response to H1N1 at this time than pigs of the H5N1 challenge control group (Table 1). Lymphocyte proliferation was partially subtype cross-reactive and the mean stimulation index (SI) to H5N1 and H5N2 was respectively 20-fold and 18-fold higher in the H1N1-immune pigs than in the challenge control pigs. Similarly, restimulation with H1N1 resulted in higher IFN-gamma secretion in the H1N1-H5N1 group (mean titre 313 pg/ml) than in the H5N1 control group (mean titre 12 pg/ml), and IFN-gamma titres in response to H5N1 or H5N2 viruses were comparable to those to H1N1. With the H5N1 challenge, the pigs of both groups developed HI and VN antibodies to H5N1. NI antibody titres to H5N1 were detected for the first time in the H5N1 challenge control group and showed a greater than 4-fold increase in the H1N1-H5N1 group. In comparison with pre-challenge values, lymphoproliferation and IFN-gamma secretion in response to all 3 viruses increased significantly in pigs of the H5N1 control group only and reached similar levels to those in the H1N1-H5N1 group.

**Discussion:** Our data demonstrate that immunity to an H1N1 swine influenza virus can partially protect pigs against infection and disease induced by a LP H5N1 AI virus. Cross-protection was clearly independent of any antibody to the viral HA, and may be mediated by an antibody to the N1 NA and/or cross-reactive cell-mediated immunity. These findings suggest that people with a solid immunity to human H1N1 influenza virus may be protected to some extent against H5N1 AI viruses, but further studies are clearly required. As an example, it remains to be seen whether cross-protection would also occur with longer time intervals between H1N1 and H5N1 virus exposures, or against H5N1 viruses with an antigenically distantly related N1. Furthermore, the H5N1 AI virus used in our study was not swine-adapted and clearly had lower replication efficiency than influenza viruses that are endemic in pigs. Further studies should therefore reveal whether increasing levels of immunity to H1N1 in humans could be a possible pandemic strategy against H5N1.

Table 1. Humoral and cell-mediated immune responses and replication of a low pathogenic H5N1 avian influenza virus in pigs previously infected with H1N1 swine influenza virus and in controls.

Group	Mean antibody titre				Mean SI		Number of pigs with virus isolation				
	HI		NI		H1N1	H5N1	Nasal	Naso-	Tonsil	Trachea	Lung
	H1N1	H5N1	H1N1	H5N1			Mucosa	pharynx			
Control	<10	<10	<10	<10	2.06	1.16	5/9	3/9	2/9	7/9	9/9
H1N1-immune	80	<10	65	44	39.13	22.70	3/10	0/10	0/10	0/10	2/10

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## SS10 MATHEMATICAL MODELING

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### SS10-1 CD8 T cell epitope recognition: broad detection through a narrow window

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Paul Thomas; A.A. High; C.A. Slaughter; P.C. Doherty

*St. Jude Children's Research Hospital, USA*

Immunodominance, the differential expansion of pathogen-specific lymphocytes, is a key feature of many CD8+ T cell responses. In influenza infection of BL/6 mice, the immunodominance hierarchy of seven epitopes is well defined in both primary and secondary responses. Previous work led to the hypothesis that peak levels of CD8+ T cell expansion are primarily a function of epitope density and precursor frequency. To measure epitope density, we generated a highly quantitative mass spectrometry protocol using isotopically labelled internal standards of each known influenza epitope peptide. This procedure allows accurate measurement of peptide loss during purification, and simultaneously, precise quantification of five peptides from a single sample. We used this technique to follow epitope presentation over a time course in infected dendritic cells. The results indicate a surprisingly broad dynamic range of epitope presentation, from hundreds of copies of peptides/cell, to >10<sup>6</sup> peptides/cell for the most abundant epitope. Over 24 hours, the absolute levels of each peptide varied widely, in some cases over three orders of magnitude, with each epitope falling into two approximate patterns: starting high and dropping, or starting low and rising. Immunodominant epitopes were typically in the former group. Epitopes corresponding to "compensating" responses, those that increase significantly when dominant epitopes are deleted, fell in the second group, peaking later in the time course. Separately, we generated a differential equations-based model of immunodominance. Fitting the data acquired thus far to the model indicates that, for secondary responses, epitope densities detected at early time points (<6 hours) contribute most to immunodominance. The success of this model indicates that under situations of high competition between several epitopes, the primary determinants of immunodominance are early time point epitope densities and precursor frequency. When lower levels of competition are present, the model predicts that the window of epitope detection lengthens, a finding consistent with data derived from ongoing studies in the lab and suggesting that the model may be predictive of in vivo responses.

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### SS10-2 Mathematical Modeling of Pandemic Influenza Containment and Control

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Ira M. Longini Jr.

*Professor*

*Program of Biostatistics and Biomathematics*

*Vaccine and Infectious Disease Institute*

*Hutchinson Research Center*

*and Department of Biostatistics, School of Public Health and Community Medicine, University of Washington, USA*

In this talk, I will describe the recent use of mathematical models for the detection, transmission and control of pandemic influenza. I will first describe basic probability models for detection of infectiousness and estimation of critical parameters for emerging pandemic influenza in clusters of households and other mixing groups. I will then expand the scope of the modeling to include wider geographical regions where containment with antiviral agents, pre-pandemic influenza vaccines and social distancing measures may still be effective for containment. Following this, I will describe mathematical models for the infection spread on a wide geographic level. This will include descriptions of what mitigation strategies may be effective to slow spread until a well-matched pandemic vaccine can be developed, manufactured and distributed.

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### SS10-3 Estimating the impact of school closure on influenza transmission from sentinel data

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Simon Cauchemez<sup>1</sup>; A.J. Valleron<sup>2</sup>; P.Y. Boelle<sup>2</sup>; A. Flahault<sup>3</sup>; N.M. Ferguson<sup>1</sup>

<sup>1</sup> *MRC Centre for Outbreak Analysis and Modelling, Department of Infectious Diseases Epidemiology, Imperial College London, UK*

<sup>2</sup> *INSERM, UMR S 707, France*

<sup>3</sup> *French School of Public Health (EHESP), France*

The threat posed by the highly pathogenic H5N1 influenza virus requires public health authorities to prepare for a human pandemic. Although pre-pandemic vaccines and antiviral drugs might significantly reduce illness rates, their stockpiling is too expensive to be practical for many countries. Consequently, alternative control strategies, based on non-pharmaceutical interventions, are a potentially attractive policy option. School closure is the measure most often considered. The high social and economic costs of closing schools for months make it an expensive and therefore controversial policy, and the current absence of quantitative data on the role of schools during influenza epidemics means there is little consensus on the probable effectiveness of school closure in reducing the impact of a pandemic. Here, from the joint analysis of surveillance data and holiday timing in France, we quantify the role of schools in influenza epidemics and predict the effect of school closure

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during a pandemic. We show that holidays lead to a 20–29% reduction in the rate at which influenza is transmitted to children, but that they have no detectable effect on the contact patterns of adults. Holidays prevent 16–18% of seasonal influenza cases (18–21% in children). By extrapolation, we find that prolonged school closure during a pandemic might reduce the cumulative number of cases by 13–17% (18–23% in children) and peak attack rates by up to 39–45% (47–52% in children). The impact of school closure would be reduced if it proved difficult to maintain low contact rates among children for a prolonged period.

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#### SS10-4 Extreme risk of influenza death among young adults: A comparative modelling study of the 1918-20 pandemic in 13 countries

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B. Finkelman<sup>1</sup>; B. Grenfell<sup>1</sup>; J.K. Taubenberger<sup>2</sup>; L. Simonsen<sup>3</sup>; S.A. Richard<sup>4</sup>; **C. Viboud<sup>4</sup>**

<sup>1</sup> Center for Infectious Disease Dynamics, Department of Biology, The Pennsylvania State University, PA, USA

<sup>2</sup> National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA

<sup>3</sup> School of Public Health and Health Services, George Washington University, Washington, DC, USA

<sup>4</sup> Fogarty International Center, National Institutes of Health, Bethesda, MD, USA

**Background:** Past epidemiological studies have shown that the 1918-20 influenza pandemic was marked by unusually high mortality among young adults. Despite the broad historical significance and contemporary relevance of this pandemic, this defining feature has not been fully confirmed on a global scale and remains biologically unexplained. Here we quantified age-specific mortality patterns associated with the 1918-20 pandemic in 13 countries in North America, Europe, Asia and Oceania, and modelled biological hypotheses potentially explaining the observed patterns.

**Methods:** First, we analyzed all-cause mortality and population data available for 12 countries by single year of age from the publicly available Human Mortality Database, 1915-23, and estimated excess mortality rates associated with the pandemic as mortality rates during 1918-20 in excess of baseline years (1915-17 and 1921-23). We also applied the same approach to national vital statistics from the US, available by 5-year age groups. To take into account differences in baseline risk of death across countries and age groups, we computed the relative risk of death, defined as the ratio of pandemic excess death rates to baseline deaths in non-pandemic years.

Second, we simulated 3 biological models potentially explaining the observed age patterns, including negative antibody enhancement, interaction with tuberculosis, and antigenic recycling. In the negative antibody enhancement model, first exposure to the influenza virus responsible for the 1889 pandemic

leads to increased mortality during the 1918-20 pandemic. In the tuberculosis interaction model, the presence of active or recently active tuberculosis predisposes an individual to death during the 1918-20 pandemic. In the antigenic recycling model, previous exposure to an A/H1 virus circulating prior to the 1889 pandemic confers partial protection from death during the 1918-20 pandemic.

To calibrate the models, we used historical data on age-specific attack rates during the 1918-20 pandemic, and simulated different age-specific case fatality rates, depending on model assumptions. We then compared the age mortality patterns obtained for the different models with those observed historically in the 13 countries studied.

**Results:** Despite variations in mortality patterns between countries, especially in the extreme age groups (infants and seniors), our study confirms a large increase in mortality risk in young adults, systematically found in all countries. Specifically, the risk of death relative to baseline non-pandemic years increased sharply at age ~ 15 years, peaked at age 28.4 years on average (range 24-31), and declined until age ~42 years. On average across all countries, adults aged 28 years had a 5.9-fold higher risk of death during pandemic years, as compared with baseline years (range 5.1-7.0). There was no difference in the age of peak relative risk between countries ( $P=0.99$ ), suggesting that biological explanations of this unusual age mortality distribution are worth further investigation.

In simulation studies, the negative antibody enhancement model captured the mortality decline in adults over age 28, but predicted high mortality in adolescents, which was not observed in historical mortality data. The tuberculosis interaction model captured both the rise in mortality in young adults and decline in older adults; however, presence of tuberculosis would need to increase influenza-related mortality by 400% in order to scale with the observed data. The antigenic recycling model most accurately captured the mortality decline in older adults, but predicted high mortality in adolescents, which was not observed in the 13 countries under study.

**Conclusion:** We confirmed on a broad geographic scale the unusual pattern of high risk of death in young adults during the 1918-20 influenza pandemic, one of the defining features of this pandemic. Normalization of pandemic excess mortality rates by baseline mortality rates produced a remarkably consistent pattern among the 13 countries, suggesting that between-country differences in pandemic mortality burden were not specific to influenza, but instead associated with underlying factors that affect baseline mortality.

We conclude that the most likely biological explanation for the observed age mortality patterns of the 1918-20 pandemic is a composite one, involving a protection effect against mortality in adolescents, combined with partial immunity in older individuals due to antigenic recycling. None of the biological hypotheses modelled could completely account for the intriguing difference in

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case fatality rates between adolescents and young adults, which could stem from age-differences in cytokine storm response or risk of secondary bacterial infections. Further experimental work is needed to address this question.



### SS10-5 Estimating the serial interval of influenza from natural infections in households

**B.J. Cowling;** V.J. Fang; S. Riley; J.S.M. Peiris; G.M. Leung

*The University of Hong Kong, Hong Kong*

**Background:** Estimates of the serial interval of human influenza infection are used directly to inform authorities as regards quarantine policy and also in parameterising mathematical models. Such empirical data are sparse and mostly come from volunteer infection experiments. Here, we describe the application of robust statistical methods to estimate the serial interval of influenza transmission in households.

**Methods:** We used natural infection data from a nested observational study as part of a large trial in which household index cases were recruited after reporting to an outpatient clinic with symptoms. Households were followed up with repeated home visits over 10 days. Influenza infection was confirmed by viral culture or RT-PCR.

**Results:** Based on a Weibull model, we used data on symptom onset times from 21 pairs of infector-infectee and estimated the mean serial interval at 3.6 days (95% bootstrap confidence interval: 2.9, 4.3 days), with a standard deviation of 1.6 days. Our statistical methods accounted for truncation bias inherent in our field study design. Non-parametric estimates, estimates based on gamma and lognormal distributions, and estimates based on different points in the natural history were not significantly different.

**Conclusions:** Our findings are generally consistent with previous field studies in households, although slightly longer than some estimates made in transmission models. If we follow inferences from airplane exposure data on the incubation period, our results suggest that the average duration of infectiousness is 2 days. A larger dataset will be available by the date of the conference and we intend to compare estimates of the serial interval for transmission in different settings, and also to investigate how the serial interval is affected by various factors such as age of the index, severity of the symptoms or the degree of viral shedding over time, any interventions that were applied, and household size and composition.

## SS11 IMMUNOLOGY

### SS11-1 Antibody quality versus quantity

**G.M. Air<sup>1</sup>;** J.Q. Feng<sup>1</sup>; X. Zhang<sup>1</sup>; L.F. Thompson<sup>1</sup>; J.A. James<sup>1</sup>

<sup>1</sup> *University of Oklahoma Health Sciences Center, USA*

<sup>2</sup> *Oklahoma Medical Research Foundation, USA*

We previously developed a strategy (Gulati et al., 2007; Gulati et al., 2005) to distinguish between antibodies against native viral surface antigens (potentially neutralizing) and antibodies directed against internal virus proteins or denatured viruses that are not neutralizing. We are applying this method to a multi-year study of vaccine effectiveness in patients with Systemic Lupus Erythematosus and comparing this against control subjects. The native virus is captured in wells coated with a detergent extract of human red cell membranes, allowing native HA to bind to sialic acids on the RBC membrane proteins. To measure antibodies against denatured proteins, the virus is captured in the same way so that the same amount of virus is present and then denatured with methanol. The amount of antibody increase after vaccination is inversely related to the level of pre-existing antibodies in all groups we have examined to date. Most subjects have quite high initial antibody levels and, while they show an increase after vaccination, the increase is low. Antibody avidity shows a higher degree of increase after vaccination, and is less dependent of the initial antibody level. Hemagglutination-inhibition results do not correlate with antibody levels however they show low correlation with Ka. Until now, none of our control subjects has been infected with influenza, so we are still unable to relate these antibody parameters to protection.

We propose an index consisting of antibody level plus antibody avidity, both measured against native HA or native virus, as a useful measure of immunity against influenza.

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### SS11-2 Influenza A H1N1 neuraminidase antibodies fail to provide cross-protection against lethal challenge with a highly pathogenic avian influenza A H5N1 infection in mice

H. Lu; H. Zeng; T. Sheu; K. Hancock; L. Gubareva; J. Katz; T. Tumpey

*Influenza Division, NCIRD, CDC, USA*

Highly pathogenic avian influenza (HPAI) A H5N1 viruses are now widespread among poultry in many countries in Asia, the Middle East, Europe and Africa. Sporadic human infections, primarily due to transmission of the virus from infected birds to humans, continue to occur with a high fatality rate. These events highlight the need for accelerated efforts to devise protective strategies against these viruses with pandemic potential. The influenza virus neuraminidase (NA) plays a role in infection-permissive immunity. Previous studies have shown that immunization with NA vaccines provided cross-protection against heterovariant influenza viruses in mice within the same NA subtype. To determine whether N1 NA-specific antibodies can provide cross-protective immunity against the HPAI H5N1 virus, we immunized rabbits with purified baculovirus-expressed recombinant NA from A/Beijing/262/95 [(H1N1), BJ/262] or NA from A/Hong Kong/483/97 [(H5N1), HK/483] HPAI viruses. In vitro cross-reactive antibody responses were evaluated by neuraminidase inhibition, single radial hemolysis (SRH), and plaque reduction assays. In vivo cross-protection of H1N1 NA-specific antibodies against HPAI H5N1 virus was evaluated in BALB/c mice. Hyperimmunization of rabbits with rNA protein generated high titers of NA-specific antibodies against the homologous viruses. BJ/262 NA-specific antibodies exhibited substantial inhibition of NA activity of the homologous virus, but failed to inhibit the NA activity of the heterologous HK/483 virus. BJ/262 NA-specific antibodies substantially reduced the plaque size and plaque number of the homologous virus when used at low serum concentrations, but only slightly reduced plaque size, but not plaque number, of HK/483 virus when a high serum concentration was used. BJ/262 NA antiserum also exhibited some reactivity against H5N1 virus in the SRH assay. However, this antiserum was ineffective at providing cross-protection against HK/483 virus in mice. Even at a very low challenge dose (3 LD<sub>50</sub>), all mice passively immunized intraperitoneally with BJ262 anti-NA serum died following HK/483 virus infection. Although passive immunization with BJ262 anti-NA serum reduced disease slightly, the antibody treatment failed to reduce the viral replication in the lungs and brains of mice. In contrast, HK483 NA-specific antibodies afforded almost complete protection against the homologous H5N1 virus. The body weight loss was diminished from 22% in untreated animals to 12% ( $p < 0.01$ ) in mice injected sequentially with the homologous anti-NA antiserum, and a concomitant 3.5-log ( $p < 0.05$ ) reduction in pulmonary virus titer and 2.1-log ( $p < 0.05$ ) reduction in brain virus titer was observed in treated versus untreated animals. Our results indicate that influenza A H1N1 BJ/262 NA-specific antibodies afforded very limited cross-protective immunity against HPAI HK/483 H5N1 virus.

### SS11-3 Analysis of antibody repertoires in H5N1 infected and vaccinated individuals using influenza whole genome phage display libraries

Hana Golding<sup>1</sup>; K. Subbarao<sup>2</sup>; A. Lanzavecchia<sup>3</sup>; C. Simmins<sup>4</sup>; S. Khurana<sup>1</sup>

<sup>1</sup> CBER, FDA, USA;

<sup>2</sup> NIAID, NIH, USA;

<sup>3</sup> Institute of Research in Biomedicine, Bellinzona, Switzerland;

<sup>4</sup> University of Oxford, Vietnam

The recent spread of H5N1 avian influenza viruses (AIV) among domestic poultry and transmission to humans has raised concerns about a potential pandemic, due to the lack of pre-existing immunity. Concerted efforts are underway to generate stockpiles of effective vaccines against AIVs in order to curtail human-to-human spread. Such vaccines must have good response rates in all high-risk populations, and elicit broad cross-clade protective mechanisms. In parallel with the vaccine development efforts, there is an urgent need to improve the analytical tools for comparing immune responses generated by different vaccine candidates. To address this need, we generated whole genome phage display libraries (GPD) expressing all the open reading frames of three avian influenza (H7N7 and two H5N1 clades; A/Vietnam & A/Indonesia) viruses. Each library contains 109-1010 phages, expressing influenza derived sequences ranging from 15-350 AA as fusion proteins with the bacteriophage pIII coat protein. These FLU-libraries are being used to map the antibody repertoires of: a) individuals exposed to avian influenza; b) broadly cross-reactive monoclonal antibodies; and c) avian influenza vaccine recipients. In avian influenza (A/Vietnam) exposed individuals, very broad antibody repertoires were identified with the discovery of novel epitopes in HA, NA (including catalytic site), M2, and several internal proteins, including PB1-F2. It was found that in general H5N1-neutralizing human mAbs (from AVI-recovered individuals) require large sequences in the N-terminal half of HA1 encompassing the receptor binding site (RBS) to form their conformational epitopes. Critical contact residues were identified, and could explain the cross-clade and reactivity of these mAbs. Preliminary evaluations with H5N1 vaccine candidates revealed a significant increase in the number of B cell epitopes elicited by adjuvanted vs. unadjuvanted vaccines. Importantly, some adjuvanted vaccines are able to generate antibodies recognizing large conformation-dependent epitopes spanning the HA1 RBS, with the expectation of having broader cross-reactivity against members of other H5N1 clades. In conclusion, the FLU-GPD analyses led to the identification of novel antibody epitopes that may contribute to protection or resolution of avian influenza infections. This approach could identify conserved sequences involved in broad cross-clade reactivity for future vaccine development and evaluations. Unique epitopes recognized by convalescent individuals may be useful for serodiagnostic assays to monitor new outbreaks of avian influenza.

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#### SS11-4 Influenza virus CTL epitopes, remarkably conserved and remarkably variable

**Guus Rimmelzwaan;** Joost Kreijtz; Rogier Bodewes; Ron Fouchier; Ab Osterhaus

*Department of Virology, Erasmus Medical Center, Rotterdam, the Netherlands*

Virus specific cytotoxic T lymphocytes contribute to the control of virus infections including those caused by influenza viruses. Especially under circumstances when antibodies induced by previous infection or vaccination fail to recognize and neutralize the virus causing the infection adequately, CTL are important and contribute to protective immunity. During epidemic outbreaks of seasonal influenza caused by antigenic drift variants and during pandemic outbreaks of influenza, humoral immunity against influenza viruses is inadequate and pre-existing CTL which are mainly directed at the relatively conserved internal proteins of the virus like the nucleoprotein and the matrix protein may provide cross-protective immunity<sup>13</sup>. Indeed most of the known human influenza virus CTL epitopes are conserved. However, during the evolution of A/H3N2 viruses, which are the major cause of seasonal influenza outbreaks, variation in CTL epitopes has been observed<sup>1,2,3,6,7,8,11</sup>. The observed amino acid substitutions affected recognition by virus-specific CTL and the human virus-specific CTL response in vitro<sup>9,12</sup>. Examples of variable epitopes and their HLA restrictions are: NP<sub>383-391</sub>/HLA-B\*2705, NP<sub>380-388</sub>/HLA-B\*0801, NP<sub>418-426</sub>/HLA-B\*3501, NP<sub>251-259</sub>/HLA-B\*4002, and NP<sub>103-111</sub>/HLA-B\*1503. In some cases, amino acid substitutions occurred at anchor residues and in other cases at T cell receptor contact residues. It is particularly interesting to note that the R384G substitution in the NP<sub>383-391</sub> epitope was detrimental to virus fitness and was only tolerated in the presence of multiple functionally compensating co-mutations<sup>4,5</sup>. In contrast, other epitopes like the HLA-A\*0201 restricted epitope from the matrix protein, M1<sub>58-66</sub>, are highly conserved despite their immunodominant nature and the high prevalence of HLA-A\*0201 in the population. A mutational analysis of this epitope indicated that it is under functional constraints<sup>10</sup>. In influenza A viruses of other subtypes, including H5N1, the M1<sub>58-66</sub> is also highly conserved. However, the M1 protein is differentially recognized by M1<sub>58-66</sub> specific CTL, which may reflect adaptation of influenza A viruses to human CTL immunity at the population level<sup>14</sup>.

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#### SS11-5 CTLs, misacylation and supercoding

**Jonathan Yewdell**

*Laboratory of Viral Diseases, NIAID*

In the beginning, there were T cells that killed histocompatible cells infected with influenza A virus. Such T cells recognized any and all of the virus gene products (then 10, now 11, but that's another story) in the form of oligopeptides bound to MHC class I molecules. The peptides are generated by proteases, with the lion's share of the work (probably) performed by the proteasome. Surprisingly, not all proteasomes are the same when it comes to generating relevant peptides. Proteasomes that degrade old proteins don't contribute much to generating class I-peptides complexes. Rather, almost all of the antigenic peptides come from proteasomes that degrade nascent proteins. This is a good idea for rapid detection of virus infected-cells but is hard to understand based on what we know about protein degradation. Odd phenomena lead to odd ideas. What if a subset of tRNAs were deliberately coupled to the "wrong" amino acid (misacylation) to generate "mutated" proteins that misfold and are rapidly degraded? What if the 151 tRNA genes encoded by the human MHC (strange but true) played a special role in this process? That's what led us to study misacylation induced by influenza A and other viruses. We found that influenza A virus does increase the amount of Met that is coupled to non-cognate tRNAs, and so do other viruses. But TLR ligands (including LPS) also do the trick. Which suggests that the misacylation may have less to do with peptide generation and more to do with an innate cell response to infection. Another odd idea: could there be an alternate genetic code ("supercoding") induced by stress where certain amino acids are replaced by others?

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## SS12 VACCINE EVALUATION

### SS12-1 New challenges for influenza vaccine development

**John M Wood**

*National Institute for Biological Standards and Control  
South Mimms  
Potters Bar, Hertfordshire EN6 3QG, UK*

Influenza vaccination offers the most effective large-scale preventative measure against seasonal and pandemic influenza and in order to have assurance about the quality of vaccines, there should be effective standardisation. Seasonal influenza vaccines are standardised internationally by well-accepted procedures, but there have been some recent developments which present significant challenges. The presentation will focus on three areas.

**Impact of cell culture vaccines:** Most of current seasonal influenza vaccines are based on the growth of influenza viruses in hens' eggs. Mammalian cells offer the potential for a more reliable and versatile substrate for both seasonal and pandemic vaccine development and such vaccines are now being licensed. Such potential must however be tempered by regulatory and safety challenges of cell culture technology.

**Influenza vaccine serology:** For research purposes and for licensing, influenza vaccines are evaluated by clinical trial, whereby immunogenicity is assessed by the presence of serum antibody. However, collaborative studies have shown that the serology assays are highly variable between laboratories, leading to attempts to standardise them. A report on EU and WHO studies will be given.

**Pandemic vaccine standardisation:** There are particular issues in attempts to standardise pandemic vaccines and the WHO has recently developed international regulatory guidance. Some of these issues will be discussed.

### SS12-2 Component-specific efficacy of trivalent influenza vaccine: sentinel surveillance to detect virus variation and impact on protection

**Danuta Skowronski<sup>1</sup>**; G. De Serres<sup>2</sup>; J. Dickinson<sup>3</sup>; M. Petric<sup>4</sup>; K. Fonseca<sup>5</sup>; H. Charest<sup>2</sup>; N. Bastien<sup>6</sup>; Y. Li<sup>6</sup>

<sup>1</sup> BC Centre for Disease Control, Canada

<sup>2</sup> Quebec National Institute of Public Health, Canada

<sup>3</sup> University of Calgary, Canada

<sup>4</sup> BC Centre for Disease Control, Canada

<sup>5</sup> Alberta Provincial Laboratory, Canada

<sup>6</sup> National Microbiology Laboratory, Canada

**Background:** Trivalent inactivated influenza vaccine (TIV) is reformulated annually to contain representative strains within two influenza A subtypes (H1N1 and H3N2) and one B lineage (Yamagata or Victoria). We describe a sentinel surveillance approach to detect new virus variants and to correlate vaccine (mis)match during the 2006-07 season with component-specific estimates of TIV efficacy.

**Methods:** TIV components for the 2006-07 season in the Northern Hemisphere included A/NewCaledonia/20/99(H1N1)-like, A/Wisconsin/67/2005(H3N2)-like and B/Malaysia/2506/2004-like (Victoria lineage) viruses. Participants were 9 years of age or older and presented an influenza-like illness (ILI) to a sentinel physician in three provinces of Canada between November 20, 2006 and April 30, 2007. Cases were participants in whom influenza was identified by PCR or isolation in cell culture; controls presented ILI but tested negative for influenza. Isolates were characterized by hemagglutination-inhibition and gene-sequencing. Odds ratios (OR) for influenza in vaccinated versus non-vaccinated persons were derived with adjustment for relevant covariates. Vaccine efficacy (VE) was estimated as 1-OR.

**Results:** 841 participants were included, among whom 20% had received the 2006-07 TIV. Influenza was detected in 337 (40%), including: 242 (72%) A/H3N2; 55 (16%) A/H1N1; and 36 (11%) influenza B. All but one A/H1N1 isolate was well-matched to the vaccine. Half of A/H3N2 isolates were strain mismatched to the vaccine, clustering equally around A/Brisbane/9/2006-like or A/Nepal/921/2006-like variants. All influenza B isolates were substantially mismatched to the vaccine at the lineage level (Yamagata). Age-adjusted VE for A/H1N1, A/H3N2 and B components was: 92% (95%CI 40%-99%), 41% (95%CI 5-63%) & 19% (95%CI 0-69%) with overall VE of 47% (95%CI 18-65%).

**Conclusions:** TIV protection varies with vaccine match to the proportionate mix of circulating influenza subtypes, strains and new variants. Component-specific VE estimates can be reliably derived annually using a sentinel surveillance platform.

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### SS12-3 Multi-state case-control study of the effectiveness of influenza vaccine in preventing laboratory-confirmed influenza hospitalizations among children aged 6-23 months during the 2005-06 and 2006-07 seasons

David Shay<sup>1</sup>; L. Sokolow<sup>1</sup>; P. Cheng<sup>1</sup>; J. Palumbo<sup>2</sup>; J. Meek<sup>2</sup>; C. Morin<sup>3</sup>; R. Lynfield<sup>3</sup>; M. Vandermeer<sup>4</sup>; A. Thomas<sup>4</sup>; K. Openo<sup>5</sup>; M. Farley<sup>5</sup>; D. Aragon<sup>6</sup>; K. Gershman<sup>6</sup>; N. Perez<sup>7</sup>; A. Reingold<sup>7</sup>; M. Mueller<sup>8</sup>; A. Craig<sup>9</sup>; W. Schaffner<sup>9</sup>; J. Bresee<sup>1</sup>

<sup>1</sup> Centers for Disease Control & Prevention, USA

<sup>2</sup> Yale Emerging Infections Program, New Haven, CT, USA

<sup>3</sup> Minnesota Emerging Infections Program, Minneapolis, MN, USA

<sup>4</sup> Oregon State Public Health Division, Portland, OR, USA

<sup>5</sup> Georgia Emerging Infections Program, Atlanta, GA, USA

<sup>6</sup> Colorado Emerging Infections Program, Denver, CO, USA

<sup>7</sup> UC Berkeley School of Public Health, Berkeley, CA, USA

<sup>8</sup> New Mexico Emerging Infections Program, Albuquerque, NM, USA

<sup>9</sup> Tennessee Department of Health and Vanderbilt University Medical Center, Nashville, TN, USA

**Background:** We assessed influenza vaccine effectiveness during 2005-06 and 2006-07 within the Emerging Infections Program, a network of state health departments that conducts surveillance for laboratory-confirmed influenza hospitalizations.

**Objective:** To estimate vaccine effectiveness for preventing hospitalizations among children aged 6-23 months.

**Methods:** Cases were children hospitalized with laboratory-confirmed influenza infection in areas of 8 states (California, Colorado, Connecticut, Georgia, Minnesota, New Mexico, Oregon, and Tennessee). Influenza was diagnosed by direct fluorescence antibody (DFA), viral culture, reverse transcription polymerase chain reaction (RT-PCR), or a commercially available rapid diagnostic test. For each case, attempts were made to enrol four age- and residence-matched controls. Case and control families were interviewed and primary care providers contacted to determine vaccination status and collect information on possible confounders, including chronic medical conditions, socioeconomic status and family demographic characteristics. Conditional logistic regression was used to estimate the effectiveness of partial and full vaccination in preventing influenza-associated hospitalization, as defined by the 2007 ACIP influenza vaccine recommendations. Children were considered immunized 14 days after receipt of each dose of vaccine. Results: Ninety-three (49%) of 191 eligible cases and 333 (40%) of 832 eligible controls were enrolled. Influenza was diagnosed by DFA in 40%, by rapid test in 50%, by viral culture in 11%, by RT-PCR in 2%, and by multiple tests in 8%. Influenza A was diagnosed in 82%, B in 14%, and type was unknown for 4%. Fifty-six percent of cases and 52% of controls were male, while 72% of cases and 80% of controls were white. Forty percent of cases and controls were aged 6-11 months, 38% were 12-17 months, and 23% were 18-23 months. Overall, 51% of study participants were not immunized, 27% were partially immunized, and only 23% were fully immunized.

After controlling for presence of a high-risk condition, very low birth weight, and insurance status, partial immunization was 27% effective (95% CI -39% to 62%) and full immunization was 76% effective (95% CI 41% to 91%) in preventing influenza-confirmed hospitalizations.

**Conclusions:** To our knowledge, this is the first study to estimate influenza vaccine effectiveness for prevention of laboratory-confirmed hospitalizations in young children. Full vaccination was ~75% effective in preventing influenza-associated hospitalizations among children aged 6-23 months. Based on data from the first two seasons of a planned three-season study, partial vaccination was less than 30% effective, and not significantly protective. It is critical to ensure that children aged 6-23 months are fully immunized, if influenza-associated hospitalizations among children are to be prevented.

### SS12-4 The effect of universal influenza immunization on mortality and health care use

J.C. Kwong<sup>1</sup>; T.A. Stukel<sup>1</sup>; J. Lim<sup>1</sup>; A.J. McGeer<sup>2</sup>; R.E.G. Upshur<sup>1</sup>; H. Johansen<sup>3</sup>; C. Sambell<sup>3</sup>; W.W. Thompson<sup>4</sup>; D. Thiruchelvam<sup>1</sup>; F. Marra<sup>5</sup>; L.W. Svenson<sup>6</sup>; D.M. Manuel<sup>1</sup>

<sup>1</sup> Institute for Clinical Evaluative Sciences, Canada

<sup>2</sup> Mount Sinai Hospital, Canada

<sup>3</sup> Statistics Canada, Canada

<sup>4</sup> Centers for Disease Control and Prevention, USA

<sup>5</sup> British Columbia Centre for Disease Control, Canada

<sup>6</sup> Alberta Health and Wellness, Canada

**Background:** In 2000, Ontario, Canada, initiated the world's first large-scale universal influenza immunization program (UIIP) to provide free influenza vaccines for the entire population aged 6 months or older. Influenza immunization increased more rapidly in younger age groups in Ontario compared to other Canadian provinces which all maintained targeted programs. We evaluated the effect of Ontario's UIIP on influenza-associated mortality, hospitalizations, emergency department (ED) services, and visits to doctors' offices.

**Methods:** Mortality and hospitalization data from 1997 to 2004 for all ten Canadian provinces were obtained from national datasets. Physician billing claims for visits to EDs and doctors' offices were obtained from provincial administrative datasets for four provinces with comprehensive data. Rates of excess, influenza-associated mortality from all causes, hospitalizations, ED services and doctors' office visits for pneumonia and influenza were estimated using Poisson regression models, controlling for age, sex, province, influenza surveillance data, and temporal trends. Influenza-associated outcomes were computed as the difference

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between observed outcomes and the model-predicted baseline in the absence of influenza activity. Changes in influenza-associated outcome rates before and after UIIP introduction in Ontario were compared to the corresponding changes in other provinces.

**Results:** After UIIP introduction, influenza-associated all cause mortality decreased more in Ontario (rate ratio=0.26) than other provinces (RR=0.43) ( $p=0.002$  for comparison). Similar differences between Ontario and other provinces were observed for influenza-associated hospitalizations (RR=0.25 vs. 0.44,  $p<0.001$ ), ED visits (RR=0.31 vs. 0.70,  $p<0.001$ ), and doctors' office visits (RR=0.21 vs. 0.53,  $p<0.001$ ). Sensitivity analyses demonstrated consistent effects using different influenza season periods, more pronounced UIIP effects when poor vaccine match seasons were excluded, and no or decreased effects on summer events and control conditions.

**Discussion:** Compared to targeted programs in other provinces, the introduction of universal influenza vaccination in Ontario in 2000 was associated with larger relative reductions in mortality, hospitalizations and visits to EDs and doctors' offices. The results of this large-scale natural experiment suggest that universal vaccination may be an effective public health measure for reducing the annual burden of influenza.

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#### SS12-5 Mass vaccination of schoolchildren and influenza outbreaks in a school

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Norio Sugaya<sup>1</sup>; S.K. Kawai<sup>2</sup>; S.N. Nanri<sup>2</sup>

<sup>1</sup> Keiyu Hospital, Japan;

<sup>2</sup> Health Center, Keio University, Japan

**Background:** It was recently decided that all children in the United States between the ages of 6 months and 18 years should be vaccinated against influenza annually. In the past, Japan's influenza control strategy was to vaccinate schoolchildren based on the theory that so doing would prevent influenza epidemics in the community. However, the Japanese government abandoned the mass vaccination of schoolchildren in 1994, mainly because of lack of evidence that mass vaccination of schoolchildren had prevented the outbreaks of influenza in schools. To demonstrate the effectiveness of the mass vaccination program of schoolchildren in schools, we investigated the relation between vaccination rates and the number of class cancellation days in one elementary school in Tokyo.

**Subjects and Methods:** About 800 pupils ranging in age from 7 to 12 years were enrolled in the school. There were 6 grades, and each grade consisted of 3 classes of 40 students each. School policy was to cancel a class whenever over 20% of students in it were absent.

Until 1994, the pupils attending the school received influenza vaccine at school twice a year, in November and again in December. We investigated the influenza vaccination rates, number of class cancellation days and absentee rates in the school during the 24-year period from 1984 to 2007.

**Results:** From 1984 to 1987 (the compulsory vaccination period), the vaccination rate at the school was 96.5%, but, in 1987, new legislation allowed parents to refuse influenza vaccination of their children, and the vaccination rate rapidly declined. From 1988 to 1994 (the quasi-compulsory vaccination period), the vaccination rate averaged 66.4%. Ultimately, the government abandoned the mass vaccination of schoolchildren in 1994, and the vaccination rate at the school dropped to an average of only 2.4% during the period 1995 to 1999 (the no-school-vaccination period). From 2000 to 2003, the vaccination rate recovered to an average of 38.9% (the low voluntary vaccination period), and during the period 2004 to 2007 rose to an average of 78.6% (the high voluntary vaccination period).

Classes were cancelled in 13 of the 24 years during the period from 1984 to 2007, and the total number of days that class was cancelled during those years ranged from 2 days to 59 days. The mean number of days on which class was cancelled was 1.25 days in the compulsory vaccination period, as opposed to 8.29 days in the quasi-compulsory vaccination period, and the number peaked at 20.5 days in the no-school-vaccination period. The mean number of days of class cancellation then decreased to 9.25 days in the low voluntary vaccination period, and to 7.0 days in the high voluntary vaccination period. In the 13 years in which class was cancelled, the main epidemic strain was influenza B virus in 6 years, influenza A (H3N2) virus in 5 years, and influenza A (H1N1) virus in 2 years. The mean absentee rate also varied inversely with the vaccination rate.

**Conclusion:** The results of this study confirmed that the mass vaccination program for schoolchildren was effective in preventing outbreaks of influenza in schools. As a result, the program reduced the opportunity for influenza to spread among young children and elderly persons. Excess deaths in Japan decreased during the period when the mass vaccination program for schoolchildren was in effect, in other words, the program protected the elderly (N Engl J Med. 2001;344:889). The discontinuation of mass vaccination of schoolchildren was responsible for an increase in incidence of influenza encephalopathy among young children (Clin Infect Dis. 2005;41:939). We conclude that during the period when the mass vaccination was compulsory, schoolchildren acted as a breakwater against influenza in the

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# SCIENCE IN PRACTICE

MON 15 SEPTEMBER 2008

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## SIP01 INCREASING THE OVERALL EPIDEMIC VACCINATION COVERAGE

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### SIP01-1 Increasing the overall epidemic vaccination coverage: the macroepidemiology of influenza vaccination

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David S. Fedson

For several years, the Macroepidemiology of Influenza Vaccination Study Group (MIVSG) has documented influenza vaccine distribution, recommendations and reimbursement in an increasing number of countries throughout the world. In 2003, 56 MIVSG countries used 275 million (M) doses, 94% of the 292 M doses distributed worldwide. By 2005, the MIVSG had grown to include 73 countries. These countries used approximately 330 M doses of seasonal vaccine. In most countries, levels of vaccine use (doses distributed/1000 total population) showed relatively little change between 2002, the year before the re-emergence of H5N1 influenza, and 2005, although large differences persisted between individual countries. However, six countries (Belgium, El Salvador, Japan, Latvia, Malta and Mexico) showed substantial increases in vaccine use over this period, with Malta's increase from 124 to 657 doses/1000 standing out among them. In a few countries, vaccine use decreased, sometimes due to supply shortages. Some form of public reimbursement for vaccination was provided in approximately 60% of the surveyed countries, and they tended to have higher levels of vaccine use compared with countries with no public reimbursement. Vaccination recommendations for risk groups showed little change compared with earlier years, although the age cut-off levels for vaccinating older adults decreased in several countries. More interestingly, by 2005, seven countries had adopted policies for vaccinating children 6-23 months of age. In the US, the upper age limit for children was extended to 5 years in 2006 and 18 years in 2008.

In 2005, nine vaccine-producing countries used 59% of all doses of seasonal influenza vaccine, but had only 12% of the world's population. Influenza vaccination is gradually increasing in many countries, especially in those with rapidly developing economies. In anticipation of the next pandemic, the global capacity to produce influenza vaccine will increase substantially within the next few years. Nonetheless, sustaining this new production capacity will be difficult without a concomitant increase in seasonal vaccination.

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### SIP01-2 influenza vaccination coverage rates in four european countries during the winter of 2007/08

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Patricia R. Blank; Thomas D. Szucs

*Institute of Social and Preventive Medicine, University of Zurich, Switzerland*

**Objectives:** The objectives of the study were to identify the level of influenza vaccination coverage in four European countries in the 2007/08 season, to understand the primary drivers of and barriers to vaccination, and to assess the major encouraging factors for vaccination and the vaccination intentions for the following winter of 2008/09.

**Methods:** Representative household surveys were performed with telephone interviews of individuals aged 14 and above. The questionnaire used in the UK, Germany, Italy and Spain was essentially the same. The research was carried out between December 2007 and January 2008.

**Results:** In the 2007/08 season, the vaccination rates in the general population remained stable in Germany (28% vs. 28% in 2006), Spain (24% vs. 22% in 2006) and Italy (23% vs. 24% in 2006). The coverage increased by four points in UK from 25% in 2006/07 to 29% in 2007/08. The proportion of individuals never vaccinated ranged between 46% in Germany and 69% in Italy. Across all four countries, the most frequent reasons for getting vaccinated was advice from a family doctor or nurse (65%), the perception of flu as a serious illness (56%) or preventing the transmission to family members or friends (44%). Having forgotten to vaccinate (40%) or not feeling concerned (27%) was the major cause for not getting vaccinated this year among those vaccinated in the season before. Individuals never vaccinated did not think they were likely to catch influenza (46%) or they had never considered it before (39%), whereas concerns of possible side effects from the vaccine were stated by 15%. Across all surveyed countries, the recommendation by the family doctor or nurse (67%) was deemed as the strongest

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encouraging factor, followed by the reason of travelling to regions with high risks of influenza (37%) and having more information regarding efficacy (33%). A total of 35% of the respondents intended to get immunized against influenza in 2008/09 (ranging from 27% in Italy to 43% in Germany).

**Conclusions:** In Germany, Italy and Spain, influenza vaccination coverage rates in the 2007/08 season varied little compared to the previous influenza season, whereas the UK indicated an increase of 4%. Our survey indicated that most vaccinated individuals were immunized because of the family doctor's recommendation. The reason for non-vaccination was mainly that people felt it unlikely they would catch the flu. Activation of the correct driving forces and dealing with barriers may, however, help to enhance coverage rates in Europe.

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### **SIP01-3 Maximizing seasonal influenza vaccination coverage**

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**Kristin L. Nichol**

*MD, MPH, MBA. Professor of Medicine, University of Minnesota and Associate Chief of Staff for Research, Minneapolis VA Medical Center.*

Seasonal influenza remains a major cause of morbidity and mortality worldwide. Vaccination represents the mainstay of prevention and control efforts, and yet vaccination rates for targeted groups are still well below established goals in many countries. In the US, annual vaccination is now recommended for 73% of the population. In 2006, only 32.3% of all targeted persons had been vaccinated, including 47.2% of high-risk persons and 64.6% of persons 65 and older. Barriers to effective vaccine delivery include ongoing misperceptions about personal risk for influenza and its complications, unfounded concerns about vaccine safety and efficacy, and lack of organized programs that facilitate convenient access to vaccine. Facilitators of effective vaccine delivery include effective education about this "bad disease and good vaccines", provider recommendation, reminders to patients, feedback to providers, and organized systems that include standing orders and other strategies to promote vaccination. Healthcare workers should also be encouraged to be vaccinated for the protection of their patients and because vaccinated healthcare workers are also more likely to vaccinate their patients. By reducing barriers and implementing effective strategies, we will enhance vaccine delivery and improve the prevention and control of seasonal influenza.

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### **SIP02 SEASONAL VACCINATION OF HEALTH CARE WORKERS**

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#### **SIP02-1 Effectiveness and cost-effectiveness of vaccinating healthcare workers against influenza, and strategies to improve uptake**

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**Rachel Jordan**

*Department of Public Health & Epidemiology  
University of Birmingham*

Influenza causes substantial annual morbidity and mortality worldwide, especially among high-risk groups, and despite targeted vaccination campaigns. Influenza vaccine has been shown to be both effective and cost-effective in preventing influenza amongst healthcare workers, and additionally is effective in reducing mortality amongst their patients. Many countries now recommend that healthcare workers be vaccinated, however the uptake is generally low (less than 25% in Europe and the UK). There is now a debate about the best method of improving uptake. In this presentation, evidence of the effectiveness and cost-effectiveness of vaccinating healthcare workers will be provided, along with an evaluation of the evidence concerning the best methods for improving uptake.

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#### **SIP02-2 The ethics of mandatory vaccination against influenza for health care workers**

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**J.J.M. van Delden et al.**

Vaccination of health care workers (HCW) in long-term care results in indirect protection of patients who are at high-risk of influenza. The voluntary uptake of influenza vaccination among HCW is generally low. We argue that institutions caring for frail elderly have the responsibility to implement voluntary programmes for vaccination against influenza of HCW. When uptake falls short, a mandatory programme may be justified. The main justification stems from the professional duty not to harm one's patient when one knows there is a significant risk of harm and the intervention to reduce this chance has a favourable balance of benefit over burdens and risks.

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**SIP02-3 National influenza immunization campaign – focus on health care workers**

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**Dietmar Walter**; Silja Wortbeg (BZgA)*Robert Koch-Institut*

The German Standing Committee on Vaccination (STIKO) recommends influenza immunization for persons aged 60 years or older, persons with special health risks and health care workers (HCWs).

Immunization of HCWs is an important step in preventing nosocomial transmission of influenza to patients, many of whom run a high risk of severe complications. Past attempts to increase low vaccination coverage in HCW have led to limited success.

In 2006 the Robert Koch-Institute (RKI) and the Federal Centre for Health Education (BZgA) launched a nationwide immunization campaign ("Wir kommen der Grippe zuvor!") to increase the coverage of target groups and to reach the WHO immunization goal of 75% coverage in risk groups by 2010. In the 2007/2008 season, tailored educational material for HCWs was sent to all hospitals and nursing homes in Germany. The evaluation of the campaign showed wide acceptance of the material but also the need for further support of local activities. Therefore in the 2008/2009 season, all German hospitals will be invited to take part in a "good practice" contest to increase the number of hospitals conducting local vaccination activities. In addition to the provision of educational material, incentives will be offered to motivate HCWs to obtain influenza vaccination.

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**SIP03 EPIDEMIC AND PANDEMIC USE OF ANTIVIRALS**

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**SIP03-1 Antiviral use in a pandemic: predicting impact and the risk of resistance**

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**Neil M. Ferguson***D.Phil., Director, MRC Centre for Outbreak Analysis and Modelling, Imperial College London, UK*

While historical clinical trial data give robust estimates of best-case impact of mass use of antivirals on transmission of influenza in a pandemic, many uncertainties remain. These centre around likely diagnostic accuracy and therefore treatment specificity, adherence and the occurrence of high-level resistance. I will first review past work on what mathematical modelling predicting antiviral treatment and prophylaxis might achieve in reducing transmission in containment or mitigation. I will then discuss what the rapid spread of the transmission-fit oseltamivir-resistant H1N1 strain across Europe in 2007-8 implies for the possible emergence of antiviral resistance in a pandemic. The uncertainties surrounding the ongoing spread of the H1N1 resistant strain remain so great as to prevent modelling of anything but illustrative scenarios regarding the evolution of resistance in a pandemic. I will therefore conclude by highlighting data needs in relation to both the current seasonal H1N1 resistant strains and monitoring of resistance in a pandemic.

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**SIP03-2**

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**J. Van-Tam***University of Nottingham, UK*

The origins and subtype of a future influenza pandemic are unknown. Although the concept of pre-pandemic vaccination is now firmly established, and human H5N1 vaccines are available to governments, these are sub-type specific and cannot at present offer 'insurance' against a wide range of possible pandemic progenitors. This being the case, the deployment of antiviral drugs will remain an important element of pandemic preparedness and response for many countries (alongside vaccines). Policy makers and strategists need to understand the evidence base for effectiveness, and possible modes of deployment at population level. Practitioners need to be familiar enough to use the drugs confidently, and local Health Service Planners need to formulate plans which ensure rapid access.

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**SIP03-3 Antivirals for Seasonal, A(H5N1), and Pandemic Influenza: Efficacy, Resistance, and New Agents**


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**Frederick G. Hayden***MD, University of Virginia School of Medicine, Charlottesville, Virginia, USA*

Chemoprophylaxis with M2 inhibitors has proven efficacy in seasonal and pandemic influenza and is a less costly option for prophylaxis if the circulating strain is susceptible. However, M2 inhibitors are not a reliable intervention currently due to widespread antiviral resistance in A(H3N2) and to a lesser extent A(H1N1) and A(H5N1) viruses, such that their use needs to be guided by knowledge of local susceptibility patterns. Early mass, geographically-targeted neuraminidase inhibitor (NAI) prophylaxis might succeed under certain conditions in containing or delaying the emergence of a pandemic, however multiple caveats apply. Inhaled zanamivir is a highly effective alternative for prevention, although unstudied as yet in human A(H5N1) infection. In pandemic influenza, long-term prophylaxis of healthcare workers should prove effective but represents an inefficient use of limited drug supplies.

Early NAI treatment with oral oseltamivir or inhaled zanamivir reduces functional disability and lower respiratory tract complications in seasonal influenza. Oseltamivir treatment also appears to reduce all-cause hospitalisations and likely mortality, including that of A(H5N1) disease, but it is unclear to what extent treatment reduces contagiousness. If available in sufficient time (rapid distribution) and quantities (stockpiling), NAI treatment could provide substantial benefits in pandemic influenza. At present, oseltamivir is the preferred agent for treatment, but the appropriate dose regimen and duration for A(H5N1) are uncertain. Highly oseltamivir-resistant N1 variants, due to an H274Y mutation, emerge during therapy, and may be associated with virological and possibly clinical failure in A(H5N1)-infected patients and immunocompromised hosts. Unexpectedly during the 2007-8 Northern Hemisphere season, such variants circulated in many countries in the absence of selective drug pressure. Another mutation N294S that confers reduced oseltamivir susceptibility, has also been recognized in some A(H5N1) patients. Viruses with either mutation retain susceptibility to zanamivir.

Future antiviral needs include an injectable agent that provides reliable drug delivery in seriously ill patients, with initial studies of neuraminidase inhibitors (IV/IM peramivir, IV zanamivir) showing good tolerability and high blood levels. Further clinical studies are in progress. Novel antiviral interventions that are presently in early clinical development include passive immunotherapy, topically applied long-acting neuraminidase inhibitors, an inhibitor of viral RNA polymerase (T-705), and an HA receptor destroyer (DAS181). New agents will provide opportunities for studying drug combinations, coping with the problem of antiviral resistance to current agents, and expanding the therapeutic repertoire for influenza management in the setting of a pandemic event.

**TUE 16 SEPTEMBER 2008**


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**SIP04 PREPANDEMIC AND PANDEMIC VACCINATION**


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**SIP04-1 Prioritizing pandemic influenza vaccination: public values and public policy**


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**Benjamin Schwartz***M.D. National Vaccine Program Office, U.S. Department of Health and Human Services*

Vaccination is an important component of a pandemic response. Because of the time required to develop pandemic vaccine and limited production capacity, only a portion of the population may be protected before or during a first pandemic wave. Targeting available vaccine can help achieve the pandemic response goals of decreasing health, societal and economic impacts. In 2006, a U.S. government interagency workgroup was established to develop recommendations for prioritization of pandemic influenza vaccine, updating earlier guidance that prioritizes vaccine primarily for healthcare workers and people at high risk of influenza complications or death (e.g., the elderly and persons with chronic medical conditions). A key part of the workgroup's process was to meet with the public and with representatives of stakeholder groups to identify their values and objectives for pandemic vaccination. Public meetings were held in Las Cruces, New Mexico, and Nassau County, New York, while a stakeholders meeting was held in Washington, DC. Over 100 people took part in each full-day meeting. Public participants were demographically diverse: many in New York were white, older and more affluent, whereas in New Mexico many were lower income Hispanics. Following educational presentations on influenza, influenza vaccines and pandemics, participants held small group discussions on their values and the potential importance of 10 proposed objectives for pandemic vaccination. After a plenary discussion, participants rated each objective on a 7-point Likert scale from "extremely important" (7) to "not important" (1), using electronic polling devices which displayed aggregate results in real-time. While all objectives rated at least as somewhat important, the same 4 objectives were identified at all meetings as most important: to protect persons critical to the pandemic response and who provide care for those with pandemic illness (mean = 6.5); persons who provide essential community services (mean = 6.0); persons who are at high risk of infection because of their occupation (mean = 5.8); and children (mean = 5.5). By contrast, protecting those who are most likely to get sick and die in a pandemic rated seventh among the ten goals (mean = 4.7). Recognizing the importance of considering multiple objectives simultaneously in prioritizing pandemic vaccination, we conducted a decision analysis where 57 population groups defined by their occupation or age and health status were scored on how well

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they met each vaccination objective and scores were weighted by the values obtained at the public and stakeholder meetings. Total weighted scores were stratified into categories of groups that provided healthcare and community support services, supported critical infrastructure, and protected homeland and national security and the general population. The workgroup then drafted a prioritization strategy proposing vaccination in tiers that simultaneously target groups in the four categories (<http://www.pandemicflu.gov/vaccine/prioritization.html>). After government consideration, the proposed strategy was vetted at additional public and stakeholder meetings, through a web-based engagement and by written comments. Electronic polling in each meeting and the web dialogue confirmed the values on which the strategy was based and the choice to target children before the elderly and those with chronic illness. Several specific suggestions proposed and supported by most participants were incorporated into revised guidance. Public values provided important input in developing public policy targeting a limited resource for pandemic response.

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### SIP04-2 Strategies for Containing or Slowing the Spread of Pandemic Influenza

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**Ira M. Longini Jr.**

*Professor, Program of Biostatistics and Biomathematics, Vaccine and Infectious Disease Institute, Hutchinson Research Center and Department of Biostatistics, School of Public Health and Community Medicine, University of Washington, Seattle, Washington USA*

We continue to face the threat of pandemic influenza due to highly pathogenic avian influenza A (H5N1) virus. Should this avian virus or another zoonotic influenza virus evolve or reassort to become readily transmissible among humans, the optimal approach is to contain the nascent strain of influenza at the source. If this fails, then the best strategy is to slow the spread until a well-matched vaccine can be made and distributed. In this talk, I will first describe methods for estimating vaccine efficacy for vaccines in general and for influenza vaccine in particular. I will then show some estimated vaccine efficacy measures for past influenza vaccine challenge studies and phase III and IV vaccine trials. Following this, I will describe large-scale stochastic simulation models to investigate the spread of a pandemic strain of influenza virus both at the source and throughout the US. We model the impact that a variety of levels and combinations of influenza antiviral agents, vaccines and modified social mobility (including school closure and travel restrictions) have on the timing and magnitude of this spread.

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### SIP04-3 Pandemic vaccination: constraining a moving target

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**A.D.M.E. Osterhaus**

*Chairman ESWI, Erasmus MC*

Medical intervention strategies in preparation for the next influenza pandemic should include the implementation of adequate surveillance programs, stockpiling of effective antivirals, and ideally vaccination with a safe and effective pandemic influenza vaccine. However, uncertainties about the eventual efficacy and safety of candidate vaccines against a still unknown influenza virus, vaccine production capacity and long response time, are crucial issues that will eventually determine the feasibility and success of a pandemic vaccination strategy. In the past decade, significant progress has been made in all these critical areas. Novel techniques to prepare vaccines that induce broader and longer lasting protection at lower doses than current seasonal influenza vaccines, have now proven to be largely effective in pre-clinical and clinical evaluations. Vaccine production capacity has increased to levels indicating that eventually a substantial proportion of the world population could indeed be vaccinated. Finally, time from the first identification of a nascent pandemic virus to the moment that the first doses of pre-pandemic or pandemic vaccine become available can now be shortened considerably. This may be achieved by implementing novel molecular techniques, like reverse genetics, and by developing continuously updated repositories of vaccine seed viruses. These should represent the moving target of avian influenza virus reservoirs from which a pandemic virus may emerge.

In conclusion, recent and ongoing scientific and technological advances in this field will allow the world to prepare effective and safe pre-pandemic and pandemic influenza vaccines in sufficient quantities and in time to mitigate the next influenza pandemic, or perhaps even nip it in the bud. Collectively we must decide what priority should be given to this unprecedented live saving opportunity in global health care.

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## SIP05 SOCIAL DISTANCING DURING A PANDEMIC

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### SIP05-1

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**Dr J. Edmunds**

*Health Protection Agency, UK*

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### SIP05-2 US community mitigation planning

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**Martin S. Cetron**

*MD. CAPT U.S. Public Health Service  
Director, Global Migration and Quarantine  
Centers for Disease Control and Prevention*

To help prepare the nations of the world for a possible global influenza pandemic, WHO and many national governments are promoting the consideration of traditional public health measures in addition to medical countermeasures and vaccines. These traditional public health measures are often referred to as non-pharmaceutical interventions, or NPIs, which include isolation and quarantine and social distancing in the workplace, community and schools, including school dismissal when appropriate. A critical factor in pandemic influenza planning is clearly articulating the goals of community mitigation. We argue for the following 3 objectives: 1) delay the temporal impact of a pandemic by postponing the peak, 2) reduce the overall and peak attack rate (flatten the epi curve), and 3) reduce the number of cumulative deaths. Such measures could potentially provide valuable time for pandemic-strain vaccine and antiviral medication production and distribution. Optimally, appropriate NPI implementation would decrease the burden on healthcare services and critical infrastructure. Howard Markel and colleagues at the University of Michigan, along with Harvey Lipman and I, recently published an historical epidemiological study of the 1918 Influenza pandemic JAMA 2007;298(6):644-654). [http://www.cdc.gov/ncidod/dq/1918\\_flu\\_supp.htm](http://www.cdc.gov/ncidod/dq/1918_flu_supp.htm) <[http://www.cdc.gov/ncidod/dq/1918\\_flu\\_supp.htm](http://www.cdc.gov/ncidod/dq/1918_flu_supp.htm)> We examined the role of NPI for epidemic mitigation in 43 cities in the continental United States from September 8, 1918 through February 22, 1919 in order to determine whether city-to-city variation in mortality was associated with the timing, duration and combination of NPI. In 1918, U.S. cities that implemented NPI earlier had greater delays in reaching peak mortality (Spearman's  $r = -0.74$ ;  $p < .001$ ), lower peak mortality rates (Spearman's  $r = 0.31$ ;  $p = .024$ ) and lower total mortality (Spearman's  $r = 0.37$ ;  $p = .008$ ). In addition, there was a statistically significant association between increased NPI duration and a reduced total mortality burden (Spearman  $r = -0.39$ ;  $p = .005$ ). These findings demonstrated a strong association between early, sustained, and layered application of

NPI and mitigating the consequences of the 1918-19 influenza pandemic in the United States. Other historical analyses of the 1918 pandemic have been published with similar findings. Additionally, models generated by investigators in the MIDAS network have offered similar conclusions using simulations from 21st century city demographic and social structure assumptions.

In the planning for future severe influenza pandemics, we conclude that NPIs should be included as critical companion measures to developing effective vaccines and medications for prophylaxis and treatment as part of the overall community mitigation strategy. In an attempt to balance benefits and adverse consequences resulting from socially disruptive public health measures and a devastating pandemic, community mitigation strategies need to be tailored to pandemic severity and implemented cautiously within a deliberate ethical framework. The USG published its interim national recommendations in February 2007 in a document entitled "Interim Pre-Pandemic Planning Guidance: Community Strategy for Pandemic Influenza Mitigation in the United States: Early, Targeted, Layered Use of Nonpharmaceutical Interventions." ([www.pandemicflu.gov](http://www.pandemicflu.gov))

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### SIP05-3 Mitigating secondary consequences of non-pharmaceutical interventions during an influenza pandemic.

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**Benjamin Schwartz**

*M.D. National Vaccine Program Office, U.S. Department of Health and Human Services*

In a severe pandemic, implementation of non-pharmaceutical interventions (NPIs) will likely reduce transmission of influenza in U.S. communities. These interventions include voluntary isolation of ill persons and quarantine of their households, dismissing children from schools and closing childcare centers, and social distancing at work and in public settings (<http://www.pandemicflu.gov/plan/community/commitigation.html>). Early and effective implementation of these measures will reduce the overall and peak pandemic attack rates while prolonging the duration of community outbreaks. Each NPI also has secondary consequences which, if unmitigated, would adversely affect families, communities, and the country. Dismissing children from school may have the greatest impact on families and communities: it would result in an estimated 11% increment in absenteeism for the duration of a community outbreak; could affect about 30 million U.S. children who receive free or low cost breakfast and/or lunch at school; and contribute to developmental

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and educational delay for vulnerable children who rely on special programs. Because of these potential impacts, dismissing children from school for the duration of a community outbreak is proposed only for a severe pandemic. Voluntary isolation and quarantine would also contribute to absenteeism, though because of their much shorter duration, impacts would be less. Isolation and quarantine could also threaten the health and safety of people who have no one to care for them or require increased levels of care, such as people who live alone, are elderly, or are chronically ill. Planning to reduce these adverse impacts is critical if NPIs are to be effectively implemented and the ratio of benefit to harm is to be maximised. Moreover, U.S. survey results suggest that while most respondents support NPIs during a pandemic and believe that they could comply, many are concerned about potential adverse consequences (Blendon R.J. et al. *Emerging Infectious Diseases*, <http://www.cdc.gov/eid/content/14/5/778.htm>). Strategies to reduce absenteeism rates include planning child-minding responsibilities, taking advantage of family and community support, and altering work practices, including the use of teleworking and flexible schedules. Development of an accurate point-of-care diagnostic test, by increasing the positive predictive value of an influenza diagnosis, would also contribute by reducing unnecessary isolation and quarantine associated with misdiagnosis of influenza. Planning by businesses for the expected impact of child-minding absenteeism can reduce the risk for delivery of essential goods and services in communities. Reducing loss of income to families may require modification of existing statutes or assistance being made available via emergency authorities. State and community-level planning is ongoing and addresses the nutritional needs of children who rely on school meals, provides support for individuals and families who are isolated or require additional care, and meets the needs of vulnerable populations. In the developing world, where availability of pandemic vaccine and antiviral is uncertain, local solutions that facilitate effective implementation of NPIs and address potential secondary consequences are particularly important.

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## SIP06 EQUITABLE USE OF INTERVENTION STRATEGIES IN A PANDEMIC

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### SIP06-1 Where are we now and what needs to be done – pandemic countermeasure in Europe

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**Professor Angus Nicoll**

*CBE, Senior Expert, Influenza Coordination  
European Centre for Disease Prevention and Control (ECDC)  
[www.ecdc.europa](http://www.ecdc.europa)*

Since the first European Commission / WHO European Region pandemic preparedness workshop in March 2005, Europe has been working hard on pandemic preparedness. Huge progress has been made and has been documented in the two reports that the ECDC wrote with Member States at the request of the Commissioner on the EU/EEA area in late 2006 and 2007. This has benefited general preparedness and there is now overdue attention being paid to seasonal influenza immunisation where Europe has seen some of the worst as well as the best performances. The last ECDC report<sup>1</sup> especially noted the many more national strengths and innovations there when compared to even 12 months earlier. However the report also noted how some EU-EEA countries were lagging behind, and that Member States with federal structures were especially challenged. Europe is not just the EU/EEA states. WHO's European Region extends across all of northern Asia, and in its countries outside the EU the main focus has been preparing for outbreaks of Avian Influenza A(H5N1) with pandemic preparedness a secondary consideration. That will have to change with the reinvigoration expected from upgraded WHO guidance coming later this year. Europe has contributed a lot to assisting WHO in preparing this new guidance. EU/EEA countries will now have to implement this following the Road Map we expect from the French Presidency. A particular challenge will be trying to close or align the many policy gaps that exist. Not easy in a Region that revels in its diversity. However work has started at the sub-regional level. The EU/EEA countries and ECDC should also assist the WHO European Region Office in implementing the new guidance in the poorer parts of the Region. The surprising emergence of oseltamivir resistance in influenza A(H1N1) in 2007-8 provided a 'dry run' for what should happen in a pandemic. In one sense Europe did well, working together and demonstrating first what turned out to be a global phenomenon. Equally though, weakness were demonstrated, especially on coordinated epidemiological approaches and data sharing. Those will not be tolerable in a pandemic. The challenge in 2009 and 2010 will now be to sustain this work in the face of political, public and media interest that will probably be diminishing. Preparing for a pandemic is like running a marathon; even if you have run 40km and don't manage the last 2km, you have failed. Collectively Europe is probably approaching the half-way marker. However

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there is a long way to go. No country as yet seems to be able to pass even ECDC's Acid Tests.<sup>2</sup> Special attention needs to be paid to integrated planning across governments and sectors, making plans operational at the local level, interoperability at the national level, stepping up prevention efforts against seasonal influenza and extending applied influenza research. ECDC still considers that a further 2 to 3 years of effort are needed for EU countries to be confident they can respond well to a pandemic – those countries now lagging behind will need to do even more.

<sup>1</sup> See via Eurosurveillance Summary <http://www.eurosurveillance.org/ew/2007/071220.asp#5>

<sup>2</sup> See <http://www.ecdc.europa.eu/pdf/Acid%20Tests.pdf>

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#### **SIP06-2 Pandemic flu vaccine preparedness: developing countries vaccine manufacturers' perspective**

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**Dr. Suresh Jadhav**

*President – DCVMN & Executive Director – Serum Institute of India Ltd.*

Pandemic influenza is a global health threat. Protecting the population by using vaccine is the most appropriate way to overcome this problem. Developed countries have an active Seasonal Influenza Vaccination Programme and therefore it is natural that the vaccine manufacturers in these countries have the infrastructure for producing a pandemic flu vaccine. The situation in developing and under-developed countries is completely different. They do not have the financial resources for a Seasonal Influenza Vaccination Programme, nor do they have manufacturers ready to undertake this in the event of unavailability on the market; furthermore, their governments do not have the proper resources and planning. The threat of pandemic in this part of the world is more serious in view of the population density and the socio-economic status, therefore these people require more attention. As a member of the Developing Countries Vaccine Manufacturers Network (DCVMN), the Serum Institute of India Ltd (SIIL) took part in the programme set up to establish vaccine manufacturing capacities in developing/under-developed countries and sponsored by the WHO and donors. Help from donor organisations and the WHO has produced extremely encouraging start-up results for this programme. This programme is expected to create manufacturing capacities in the developing world, which would provide the people in these countries with access to pandemic influenza vaccine as and when required.

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#### **SIP06-3 The industry contribution to the equitable availability of vaccines in a pandemic situation**

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**Luc Hessel**

*European Vaccine Manufacturers*

The priority challenge of pandemic preparedness for the vaccine industry is to deliver as much vaccine as quickly as possible and at the most appropriate time. To this end, vaccine manufacturers have successfully developed antigen-sparing strategies and planned a significant increase in production capacities that could potentially solve the pandemic vaccine supply issue and make pre-pandemic strategies a reality. Ensuring equitable availability of vaccines is the other major challenge for all stakeholders involved in this endeavour. It relies on the proper analysis of the key elements that would meet scientific, regulatory, public health policy, and industrial needs. In this respect, two main strategies have been proposed: 1) use of vaccines prepared from avian strains with a pandemic potential to protect against the first pandemic wave. Such "pre-pandemic" H5N1 vaccines have been shown to confer cross protection against drift variants of the virus; 2) *stricto sensu* pandemic vaccines for use during the pandemic period, prepared from the pandemic strain after it has been isolated and characterised. The successful implementation of these approaches relies on establishing stockpiles of pre-pandemic vaccines and on securing supplies of pandemic vaccines. Processes to ensure the development, acquisition and deployment of pre- and pandemic vaccines at both international and national levels are essential to manufacturers in planning their manufacturing capacities. Appropriate contractual agreements between industry and governments or international institutions represent the best way to address such challenges and allow vaccine manufacturers to meet national and international demands in accordance with global public health needs. They should be based on reasonable forecasts relevant to specific vaccination strategies, existing regulations and public health infrastructures.

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## SIP06-4 Access to antiviral stockpiles for pandemic use – an industry perspective

**Dr David Reddy**

*Pandemic Influenza Taskforce Leader, F.Hoffmann-La Roche Ltd, Switzerland*

Global pandemic preparedness is a critical element for the protection of global society and economy. Influenza pandemics have occurred approximately every thirty years. There have been three pandemics in the 20th century, the last of which originated in Hong Kong in 1968-1969 and resulted in around 1 million deaths. According to the World Health Organization (WHO), the next pandemic is imminent, and it is estimated that 2 to 7 million people worldwide will die during this pandemic.<sup>1,2</sup>

While much has already been achieved, the issue of pandemic preparedness is not receiving the consistent attention and priority it needs from governments, international agencies, the business community, and society as a whole. In addition, while an integrated approach to pandemic planning would provide greatest benefit, agencies, governments and the business community are currently not uniformly working together across continents in a coordinated manner to prepare for this global public health threat. Pandemic influenza needs to be appreciated as not just a government public health emergency but as a potential civil emergency with potentially serious global impact on human health and well-being, the economy and major social infrastructure.

Stockpiling antivirals is one important element of a pandemic preparedness plan and the focus of this presentation. The need to ensure fair and timely access to stockpiles / appropriate medications during a pandemic will be a significant challenge to public health care systems. It is important to note that only 85 governments worldwide have antiviral stockpiles and that these cumulative stockpiles are sufficient to treat less than 5% of the world's population. Furthermore, to maximize efficacy, antiviral therapy should be initiated as rapidly as possible (within 2 days) after symptom onset. If this cannot be achieved, much of the investment in stockpiles will not be maximized to the full benefit.

In the current pre-pandemic period, Roche, the manufacturer of the antiviral Tamiflu, has filled orders placed for Tamiflu by governments, agencies or corporations on a "first come, first served" basis with governments and international agencies receiving first allocations, followed by the private sector once sufficient supplies were available. However, a stockpile alone does not ensure an adequate response to an outbreak. Comprehensive strategic pandemic preparedness plans at the global, regional and national levels must be put in place, as well as operational and logistic plans to ensure the effective implementation of these strategic plans.

Once a pandemic is declared by the WHO, antiviral manufacturers will be called upon to produce more supplies and upscale a complex manufacturing process. Based on current stocks,

Roche could start packaging existing Tamiflu capsules and production of new capsules from existing Active Pharmaceutical Ingredient (API) within weeks. However, since the lead time for new production is around 6 to 9 months, it will not be possible for Roche to respond immediately to a surge in demand by governments or corporations looking to purchase Tamiflu.

A further consideration regarding stockpiles is the fact that matched vaccines will not be available at the time of outbreak of a pandemic and only limited amounts of pre-pandemic vaccines will be available. In the first few months of a declared pandemic, antivirals and social distancing will therefore represent the principle tools available to treat and prevent the spread of the virus. Hence, having sufficient antiviral stockpiles in place becomes even more important. Therefore, in line with guidance published by the WHO, the only way to ensure that there will be sufficient supplies of antivirals at the time of the outbreak of a pandemic is to stockpile in advance. Once a pandemic is declared by the WHO, Roche will fill orders placed for Tamiflu in the following order:

- 1) Delivery of WHO Rapid Response stockpile donated by Roche to the WHO will be the first priority.
- 2) Fulfilment of existing pandemic orders, from both government and other groups.
- 3) Increase rapid response effort for containment in collaboration with WHO and other international agencies.

In summary, the scope of a pandemic will be such that the only way to address it is through comprehensive advanced preparation from stakeholders and leaders across all sectors of society. Antiviral drug manufacturers have and will continue to play a key role in pandemic preparedness.

1 World Health Organization. Ten things you need to know about pandemic influenza. October 2005. Available at <http://www.who.int/csr/disease/influenza/pandemic10things/en/index.html>

2 World Health Organization. Avian influenza: assessing the pandemic threat. January 2005. Available at <http://www.who.int/csr/disease/influenza/H5N1-9reduit.pdf>

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# **POSTER PRESENTATIONS**

## **Abstracts**

- 1 virus host interaction/pathogenesis/transmission
- 2 clinical impact and diagnostic approaches
- 3 virus structure and replication
- 4 vaccines: current and novel approaches
- 5 disease surveillance and socio economics
- 6 antivirals and resistance
- 7 genetic and antigenetic evolution
- 8 animal influenza and ecology
- 9 mathematical modelling
- 10 immunology
- 11 clinical and epidemiological vaccine evaluation

## 1 VIRUS HOST INTERACTION/PATHOGENESIS/TRANSMISSION

1-001

### Alterations in caspase cleavage motifs of NP and M2 proteins attenuate virulence of avian influenza virus

**Zhirnov, Oleg<sup>1</sup>**; Klenk, H.D.<sup>2</sup>

<sup>1</sup>D.I. Ivanovsky Institute of Virology, Russian Federation; <sup>2</sup>Institute of Virology Philipps University of Marburg, Marburg 35043, Germany

Influenza virus proteins NP and M2 carry caspase cleavage motifs at the N- and C-terminal regions, respectively. Alteration of these motifs in avian influenza virus A/FPV/Rostock/34 (H7N1) was generated by using a reverse genetics approach. Mutation ETG16→ETD restoring caspase cleavage site in FPV virus NP (NPgd) permitted its cleavage by caspases in infected cells, like the human virus NP possessing a cleavable ETD caspase site. FPV mutant virus NPgd was able to replicate in cultured cells and chicken eggs and retained Gly16→Asp mutation during passages. This mutant virus had reduced virulence in chickens comparatively to a wild-type recombinant virus (WTR). The recombinant virus generated by a deletion mutation in a caspase cleavage site (M2nn) in a cytoplasmic domain of M2 was shown to lack intracellular caspase-dependent cleavage of M2 and retain replication ability in cultured cells and chicken eggs. The WTR virus killed chickens during the few days with an input dose as low as 1-5 p.f.u., whereas the mutant M2nn was found to possess a low virulent phenotype and did not kill chickens with massive infection doses, 10,000 p.f.u. and higher. The WTR virus replicated in chickens and effectively disseminated in the body through the viremia whereas the replication of NPgd and M2nn in chickens was attenuated and viremia was observed at low level and non-regularly. A single injection of minimal dose of either NPgd or M2nn recombinant viruses induced high titers of anti-virus antibodies in chickens protecting them against a field FPV virus. These data suggest that natural caspase cleavage motifs in NP and M2 play a role in pathogenesis of influenza infection, while their alteration not restricting the replicative ability of the virus reduces its virulent potential. Finally, caspase cleavage motifs in viral proteins can be considered as a novel target for designing live vaccines.

1-002

### Mechanisms and consequences of differential IFN induction by influenza A viruses

**Hartgroves, Lorian<sup>1</sup>**; Johnson, B.<sup>1</sup>; Hayman, A.<sup>2</sup>; Linton, S.<sup>1</sup>; Howard, W.<sup>1</sup>; Barclay, W.<sup>1</sup>

<sup>1</sup>Imperial College, UK; <sup>2</sup>University of Reading, UK

Human influenza A viruses, like most viruses, have strategies for counteracting the host type I interferon (IFN) response, but their inhibition of interferon induction is not absolute. We previously found considerable variation amongst a panel of influenza A viruses in the levels of IFN they induced in mammalian cells [1, 2]. In a human epithelial cell line (A549), we know that the degree of IFN modulation and induction by these viruses are dependent on virus replication and are genetically encoded. However, it does not map to virus genes previously implicated in control of IFN induction. Firstly, a corresponding panel of cloned NS1 proteins expressed alone or in an isogenic human influenza virus genetic background were all equally adept at controlling IFN-beta induction [2]. Furthermore, A549 cell but not pig macrophage [3] induction did not map to the surface antigens HA or NA (2). We now present studies of the kinetics of replication, indicated by the accumulation of viral RNAs and proteins, of a virus that only poorly controls interferon induction. Additionally, by studying the effects of drugs that inhibit replication at different stages of the cycle, we attempt to describe the viral motif that is recognized by the host pattern recognition receptors such as RIG-I. Moreover, we implicate early events in the replication cycle as key in determining the outcome of interferon induction: we show that positive feedback via autocrine and paracrine responses can in some cases upregulate the RIG-I and MDA-5 pathogen sentinels to such an extent that the NS1 interferon antagonist is overwhelmed. The biological consequences of moderate cytokine induction by naturally occurring viruses have been tested in-vivo in both ferrets and mice. The data illustrates that, in some cases, engineered viruses which are less able to control interferon induction, but remain largely uncompromised in their replication, can lead to enhanced disease.

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3. Seo, S.H., Webby R., R.G. Webster. No apoptotic deaths and different levels of inductions of inflammatory cytokines in alveolar macrophages infected with influenza viruses. *Virology*, 2004. 329(2): p. 270-9.

### Human and avian influenza A viruses strongly modify ultrastructure and molecular composition of the host human cell nucleolus

**Rosa Calatrava, Manuel<sup>1</sup>**; Moules, V.<sup>2</sup>; Josset, L.<sup>1</sup>; Bouscambert, M.<sup>1</sup>; Ferraris, O.<sup>1</sup>; Frobert, E.<sup>1</sup>; De Chasse, B.<sup>3</sup>; Hay, A.<sup>4</sup>; Morfin, F.<sup>1</sup>; Diaz, J.J.<sup>5</sup>; Lina, B.<sup>1</sup>

<sup>1</sup>Virologie et Pathologie Humaine FRE 3011 CNRS-UCBL, France; <sup>2</sup>V, France; <sup>3</sup>IMAP INSERM-UCBL-HCL U851, France; <sup>4</sup>NIMR, UK; <sup>5</sup>UMR 5534 CNRS-UCBL, France

Influenza viruses are responsible for recurrent annual epidemics causing acute febrile respiratory illness. Moreover, they constitute a major threat to public health since they could lead to the emergence of potentially pandemic new variants. These enveloped viruses contain eight single strand negative RNA segments and most of their infectious cycle occurs within the host cellular nucleus. Both viral transcription and replication are associated with host nuclear machinery and many interactions between viral proteins and nuclear components occur during the time course of infection. However, due to the fact that influenza has never constituted tools for studying fundamental molecular mechanisms of the cell, not much data are available concerning ultrastructural and molecular relationships between influenza viruses and human host nuclear domains. Recent publications have mentioned a dynamic nucleolar localization of viral nucleoprotein (NP), early in infection. Moreover, mutations suppressing the nucleolar addressing of NP, result in an abortive viral cycle (Ozawa M. *et al.*, J. of Virology. 2007. Vol. 80, p. 30-41). Although NP is considered as a key adapter molecule between viral genome and host cell machinery, the biological significance of these events needs to be elucidated. Furthermore, the Non Structural protein NS1 was characterized as an interacting partner of nucleolin, a major nucleolar component (Murayama R. *et al.*, Biochem. Biophys. Res. Communications. 2007. Vol. 362 p. 880-5). Altogether, these results suggest a likely interplay between influenza and the host nucleolus. Nucleoli are known to be the site of ribosomal RNA (rRNA) transcription, processing, and assembly into the ribosomal subunits. In addition, nucleoli are dynamic structures involved in additional non-conventional roles including cell cycle regulation and cellular stress responses (Boisvert *et al.* Nat. Rev. Mol. Cell Biol. 2007. Vol. 8 p. 574-85). Furthermore, it is now well established that many other viruses, with nuclear replication, induce important remodeling of the ultrastructure, composition and dynamic of nucleoli, and that these modifications are required for optimal infection (Hiscox J.A., Nature Rev. Microbiol. 2007. Vol. 5, p. 119-127). In this context, we hypothesized that host nucleolus could play a crucial role in determining the outcome of influenza infections. We then explored the potential impact of influenza viruses on the ultrastructure and molecular composition of nucleolus. For this purpose, human epithelium A549 cells were infected with relevant human (A/New

Caledonia/20/99, H1N1 and A/Moscow/10/99, H3N2) and avian (A/Turkey/582/2006, H5N1; A/Finch/England/2051/94, H5N2 and A/chicken/Belgium/2003, H7N7) viruses and analysed at several times post-infection by electron microscopy and confocal laser scanning. Our results show that all these influenza type A viruses induce a strong remodelling of nucleolar morphology and a dynamic delocalization of several major constitutive nucleolar markers. These events probably imply interaction between viral nucleoprotein and host endogenous nucleolin which we characterized by a GST pull down assay. Finally, a complementary approach using reverse genetics confirmed that influenza type A viruses have a "nucleolar experience" for performing effective and optimal viral replication.

### Experimental transmission of Italian H7N1 HPAI in the mouse model

**Cattoli, G.<sup>1</sup>**; Toffan, Anna<sup>1</sup>; Michela, R.<sup>1</sup>; Shinya, K.<sup>2</sup>; Viale, E.<sup>1</sup>; Cilloni, F.<sup>1</sup>; Bertoli, E.<sup>1</sup>; Ormelli, S.<sup>1</sup>; Marciano, S.<sup>1</sup>; Milani, A.<sup>1</sup>; Kawaoka, Y.<sup>3</sup>; Capua, I.<sup>1</sup>

<sup>1</sup>Istituto Zooprofilattico Sperimentale delle Venezie, Italy; <sup>2</sup>International

Center of Medical Research and Treatment (ICMRT), School of Medicine, Kobe University, Japan; <sup>3</sup>International Research Center for Infectious Diseases, Japan Science and Technology Agency, Japan

**Introduction:** It has been demonstrated that selected Italian H7N1 Highly Pathogenic Avian Influenza (HPAI) viruses are able to replicate in experimentally infected mice causing disease and mortality (1). These findings are likely to be related to the occurrence of specific mutations in the viral genome, such as PB2 E627K. Although mice show a pattern of receptors in the upper respiratory tract, which makes this species ideal for evaluating a possible adaptation of avian influenza viruses in non-avian species (2), no experimental studies concerning transmissibility of avian influenza viruses have been reported in this species. The aim of this study was to evaluate the transmissibility of lethal Italian H7N1 HPAI viruses in the mouse model.

#### Material and methods:

**Viruses.** We selected two Italian HPAI H7N1 viruses: A/ostrich/It/2332/00 (OS/2332) displaying a lysine (K) in position 627 of the PB2 gene, and A/ostrich/Italy/984/00 (OS/984) with glutamic acid (E) in the same position. These viruses were classified as highly pathogenic for mice (OS/2332 LD<sub>50</sub>101,7) and of intermediate pathogenicity respectively (OS/984 LD<sub>50</sub> value between 103 and 106) (3).

**Mouse experiment.** Female BALB/c mice of 6-8 weeks of age were divided into 3 different groups of 30 each. Ten mice per group were infected intranasally with 50 µl of a solution containing 10

## POSTER PRESENTATIONS

LD<sub>50</sub> of each virus (Group 1-2332/00 and Group 1-984/00). 24 hours post-infection, all the infected mice were moved to a clean cage together with a group of ten sentinels (Group 2-2332/00 and Group 2-984/00). A second group of 10 sentinels was put in the same cage but separated from Group 2 sentinels by a net (Group 3-2332/00 and Group 3-984/00). Ten uninfected mice were used as a negative control group. All the animals were observed twice a day for clinical signs. Organs (lung and brain) of every mouse that died during the experiment were collected and tested by means of quantitative real-time RT-PCR (qRRT-PCR), histopathological examination and immunoistochemistry (IHC). Similarly, organs of sentinels found dead or euthanized were collected and examined.

**Results:** The uninfected control group did not show any clinical signs throughout the duration of the experiment. Mice infected with both OS/2332 and OS/984 (G1) presented clinical signs, which appeared earlier in mice infected with OS/2332. These symptoms were only nervous at first but later were accompanied by respiratory signs. All mice infected with OS/2332 died within day 7 post-infection (pi), while animals infected with OS/984 died within day 10 pi. All infected mice tested positive for qRRT-PCR. Eight out of ten sentinels of the Group 2-2332/00, in direct contact with the infected mice, died, whereas only two sentinels (Group 2-984/00) in contact with OS/984 infected mice were found dead. Brains and lungs were tested and yielded positive results either by RRT-PCR or IHC.

Among the Group 3 sentinels, only one mouse was found dead in the Group 3-2332/00 and virus was detected in both lung and brain.

**Discussion and conclusions:** Our preliminary results demonstrate that Italian H7N1 HPAI viruses could transmit from infected mice to contact sentinels. However OS/2332 and OS/984 viruses exhibited a different pattern of infection and transmission. OS/2332 showed a higher mortality rate and an earlier onset of clinical signs compared to OS/984 and it was transmitted to a significantly higher number of mice. Transmission likely occurred by contact with upper respiratory tract secretions, as no virus has been detected in the intestinal tract of infected mice (data not shown).

Further studies are in progress to understand the molecular mechanisms involved in transmission.

**Acknowledgements:**

This work was supported financially by the EU funded project FLUPATH (Contract n°044220) and by the Italian Ministry of Health project RF IZSVE 2004.

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1-005

## Development of an influenza pseudovirus based assay for the identification of entry inhibitors by phage display

**Haarmann, Thomas<sup>1</sup>**; Meroz, D.<sup>2</sup>; Ben-Tal, N.<sup>2</sup>; Zevgiti, S.<sup>3</sup>; Sakarellos-Daitsiotis, M.<sup>3</sup>; Pleschka, S.<sup>4</sup>; Planz, O.<sup>5</sup>; Rimmele, M.<sup>6</sup>; von Laer, D.M.<sup>1</sup>; Dietrich, U.<sup>1</sup>

<sup>1</sup>Georg-Speyer-Haus, Germany; <sup>2</sup>Tel Aviv University, Israel; <sup>3</sup>University of Ioannina, Greece; <sup>4</sup>Justus-Liebig-University, Germany; <sup>5</sup>Friedrich-Loeffler-Institute, Germany; <sup>6</sup>RiNA GmbH, Germany

The threat of a major human influenza pandemic, in particular from highly pathogenic avian influenza viruses (HPAIV) like H5N1, has emphasized the need for new antiviral drugs and therapeutic strategies to combat these pathogens.

We have constructed retroviral virus particles pseudotyped with either human or avian influenza hemagglutinin, neuraminidase and matrix proteins in different combinations and investigated their infectivity in a single-round infection assay. The assay is based on the transmission of a marker gene (GFP) to target cells and thereby directly dependent on the function of the envelope proteins enabling the virus to enter the host cell. Therefore, this assay can be ideally used to analyze potential entry inhibitors.

Additionally, we expressed the hemagglutinin receptor-binding domain (RBD) of human and avian influenza viruses. The pseudotyped viruses, the RBDs and parts of the HA2 domain are used as targets in phage display library screenings. This method allows us to directly identify peptides interfering with the entry process thereby potentially inhibiting viral entry. First results of these screenings will be presented.

To further investigate the specificity and affinity of the selected viral entry inhibitors we developed a solid-phase immunoassay where we use sialic acid coupled to an artificial sequential oligopeptide carrier (SOC). This technique enables us to perform these assays in a small-scale microplate format.



### Contribution of viral polymerase proteins to HA-induced Raf/MEK/ERK signal cascade

**Marjuki, Henju<sup>1</sup>**; Yen, H.L.<sup>2</sup>; Franks, J.<sup>2</sup>; Webster, R.G.<sup>2</sup>; Pleschka, S.<sup>3</sup>; Hoffmann, E.<sup>2</sup>

<sup>1</sup>St. Jude Children's Research Hospital, USA; <sup>2</sup>St. Jude Children's Research Hospital, USA; <sup>3</sup>Institute for Medical Virology, Germany

Influenza viruses replicate within the nucleus of infected cells. Viral genomic RNA, three polymerase subunits (PB2, PB1, and PA), and the nucleoprotein (NP), form ribonucleoprotein complexes (RNPs) that are exported from the nucleus late during the infectious cycle. The virus-induced Raf/MEK/ERK (MAPK) signal cascade is crucial for efficient virus replication. Any blockade of this pathway delays RNP export and reduces virus titers. Hemagglutinin (HA) accumulation and its close association with lipid rafts activate ERK and enhance localization of cytoplasmic RNPs. We studied the induction of the MAPK signal cascade by two seasonal human influenza A viruses A/HK/218449/06 (H3N2) and A/HK/218847/06 (H1N1) that differed substantially in their replication efficiency in tissue culture. Infection with H3N2 virus, which replicates efficiently, resulted in higher HA expression and its accumulation on the cell membrane, leading to substantially increased activation of MAPK signaling compared to that caused by the H1N1 subtype. More H3N2-HAs were expressed and accumulated on the cell membrane than was the case with H1N1-HAs. Viral polymerase genes, particularly H3N2-PB1 and H3N2-PB2, were shown to contribute to increased viral polymerase activity. Applying plasmid-based reverse genetics to analyze the role of PB1 protein in activating the HA-induced MAPK cascade showed that recombinant an H1N1 virus possessing the H3N2-PB1 (rgH1N1/H3N2-PB1) induced greater ERK activation, resulting in increased nuclear export of the viral genome and higher virus titers. We conclude that enhanced viral polymerase activity promotes the replication and transcription of viral RNA leading to increased accumulation of HA on the cell surface and thereby resulting in an upregulation of the MAPK cascade and more efficient nuclear RNP-export as well as virus production.



### Asparagine 631 variants of the Chicken Mx protein do not inhibit influenza replication in primary chicken embryo fibroblast cells or in vitro surrogate assays

**Benfield, Camilla<sup>1</sup>**; Lyall, J.W.<sup>1</sup>; Kochs, G.<sup>2</sup>; Tiley, L.S.<sup>1</sup>

<sup>1</sup>University of Cambridge, UK; <sup>2</sup>Department of Virology, University of Freiburg, Germany

Whether chicken Mx inhibits influenza virus replication is an important question with regard to strategies aimed at enhancing influenza resistance in domestic flocks. The Asn631 polymorphism of the chicken Mx protein found in the Shamo (SHK) line has been previously reported to be crucial for the antiviral activity of this highly polymorphic chicken gene. Our aims were to determine whether cells from commercial chicken lines containing Asn631 alleles were resistant to influenza virus infection and to investigate the effects that other polymorphisms might have on Mx function. Unexpectedly, we found that the Asn631 genotype had no impact on multi-cycle replication of influenza (A/WSN/33) in primary CEF lines. Furthermore, the Shamo (SHK) chicken Mx protein did not inhibit minireplicon systems based on influenza A/PR/8/34 (H1N1), A/Turkey/England/50-92/91 (H5N1) or Thogoto viruses, even when artificially retargeted to the nucleus. Virus infection of 293T cells transfected with Mx expression plasmids also demonstrated that Shamo (SHK) Mx had no inhibitory effect on influenza (A/PR/8/34) gene expression. Our findings indicate that Asn631 chicken Mx alleles lack anti-influenza activity in primary chicken cells and that the Shamo (SHK) allele is not active against any of the virus strains we tested in our assays. Only in vivo challenge studies will definitively confirm the antiviral potential of different chicken Mx alleles and these should take precedence over efforts to influence the frequency of Asn631 alleles in commercial chicken populations at the present time.

## POSTER PRESENTATIONS

1-008

### Pathogenesis of a Highly Pathogenic Avian Influenza (HPAI) H5N1 virus in Pekin ducks of varying ages infected experimentally

**Londt, Brandon;** Nunez, A.; Russell, C.; Banks, J.; Alexander, D.J.; Brown, I.H.

Veterinary Laboratories Agency (VLA), UK

The affect of host age on the dissemination of H5N1 virus in organs and tissues of infected Pekin ducks, was studied by infection of individuals in three age-matched groups (n=20), 4, 8 and 12 weeks old (wo), with  $10^6$  EID<sub>50</sub>/0.1ml of HPAI A/turkey/Turkey/1/05 (H5N1, clade 2.2), distributed equally by the intranasal and intraocular routes. Daily, birds were monitored clinically, and cloacal and oropharyngeal swabs were collected, before three birds from each group were randomly selected and humanely euthanized for post-mortem examination. Tissue samples were collected for examination by real-time RT-PCR (RRT-PCR), histopathology and immunohistochemistry (IHC) analyses. Severe clinical signs, including in-coordination and torticollis were observed in the 4wo and 8wo groups resulting in 100% mortality by 5dpi. Mild clinical signs were observed in the 12wo group with no mortality. RRT-PCR and IHC results demonstrated the systemic spread of H5N1 virus. Variations in level and temporal dissemination of virus within tissues of older ducks, and the presence of the virus in brain and heart tissues were observed and coincided with the appearance of clinical signs preceding death in younger birds. These results are consistent with reports of the natural infections of wild birds and poultry possibly indicating an age-related association with dissemination and clinical outcome in wild ducks following infection with H5N1 HPAI virus.

1-009

### Mutations in the nuclear export signal of NS1 regulate infectivity of a highly pathogenic avian influenza virus

**Keiner, B.;** Mänz, B.; de Vries, M.; Klenk, H.D.

Institute of Virology, Germany

The non-structural protein 1 (NS1) of influenza A viruses is known to be a multifunctional protein that acts as a strong interferon antagonist in infection. Interestingly, in an outbreak of avian influenza A/H7N1 viruses among domestic birds in Italy in 1999/2000, all highly pathogenic isolates possessed two mutations (AA-position 136 and 139) in a proposed nuclear export signal (NES) and a C-terminal deletion of 6 amino acids in the

NS1 protein compared to the sequence of their low pathogenic ancestors. In order to evaluate whether the mutations in the NS1 affect pathogenicity, we generated highly pathogenic recombinant viruses which contain the NES mutations and/or the C-terminus of NS1 of a low pathogenic virus. These mutants showed reduced virus titers in chicken embryo cells and restricted spread of infection in chicken embryos compared to the highly pathogenic wild-type virus. Immunofluorescence analysis revealed that NS1 with the NES of the highly pathogenic viruses was predominantly found in the cytoplasm, whereas NS1 with the C-terminal truncation showed strong nucleolar accumulation. Enhanced nuclear export of NS1 was paralleled by a complete shut-down of interferon- $\alpha$  production in infected cells, suggesting that this mechanism is responsible for the improved virus growth of the highly pathogenic variant.

1-010

### A highly pathogenic avian influenza subtype H5 virus displays a higher affinity to "human-like" linked sialic acid than "avian-like"

**Heider, A.<sup>1</sup>;** Mochalova, L.<sup>2</sup>; Lehmann, H.<sup>1</sup>; Harder, T.<sup>3</sup>; Bovin, N.<sup>4</sup>; Schweiger, B.<sup>1</sup>

<sup>1</sup>Robert Koch-Institute, National Reference Centre for Influenza, Berlin, Germany; <sup>2</sup>Engelgardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, Russia; <sup>3</sup>Institute of Diagnostic Virology, Friedrich-Loeffler-Institute, Greifswald-Insel Riems, Germany; <sup>4</sup>Shemyakin Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russia

Recent human infections caused by highly pathogenic avian influenza viruses (HPAIV) emphasize an urgent need for the assessment of factors that allow adaptation of avian viruses to humans. We have conducted a comparative study of the hemagglutinin (HA) receptor binding specificity of a number of HPAIV and low pathogenic avian influenza viruses (LPAIV) of H5N1, H5N9, H5N6, H5N2, H7N7 and H9N2 subtypes by testing the direct binding of whole viruses to synthetic analogs of natural influenza virus receptors, biotin-labeled sialylglycoconjugates. This simple and sensitive method allows a quantitative investigation not only of the receptor preferences for  $\alpha 2,3$ - or  $\alpha 2,6$ -linked sialic acid, but also detects fine differences in HA specificity, such as fucosylation or sulfatation of the terminal trisaccharide or carbohydrate core differences.

We have identified an avian strain of subtype H5 which showed the same binding to oligosaccharide (OS) with  $\alpha 2,6$ -linked sialic acid ("human-like"-linked) and to OS with  $\alpha 2,3$ -linked sialic acid ("avian-like"-linked). Sequence analysis of the HA gene revealed a mutation S227N in the HA receptor binding site.

The data obtained for other HPAI viruses show a high affinity level of the HA to OS with  $\alpha 2,3$ -linked sialic acid and in contrast, all LPAIV compared to HPAIV were characterised by a lower (2-6-times) HA affinity to OS with "avian-like"  $\alpha 2,3$ -linked sialic acid. Because the HA and the neuraminidase (NA) recognize the same molecule, sialic acid, we have also analysed the correlation between the HA receptor specificity and the NA substrate specificity. The investigation of the OS specificity of the NA at the tri- and tetrasaccharide level was conducted by an assay using the same set of OS as substrates, but modified with fluorescent label. The NA specificity of LPAIV to OS substrates with  $\alpha 2,3$ -linked sialic acid was significantly (to 10 times) lower than those of HPAI viruses.

In summary, this study presents for the first time combined data on HA and NA specificity of HPAIV and LPAIV. HPAIV were characterised by a high HA and NA specificity to  $\alpha 2,3$ -linked sialic acid whereas a lower specificity was typical for LPAIV. Moreover, we have identified one subtype H5 HPAIV with high receptor specificity to "human-like" OS. A combination of both methods allows for a rapid monitoring of changes in the OS specificity of both HA and NA. Correlation of these data with mutational analyses of the glycoprotein genes promise to be powerful tool for the prediction of new pandemic strains.

1-011

### Multiple organ spread of human influenza A viruses of H3 and H1 subtypes in mice

*Fislova, T.; Rajcani, J.; Mucha, V.; Vareckova, E.; Kostolansky, F.*

*Institute of Virology, Bratislava, Slovakia*

Our pathogenetical studies of three mouse adapted epidemic human influenza A viruses showed that studied influenza viruses can reach organs outside the respiratory tract. Mouse adapted influenza A viruses A/Dunedin/4/73 (H3N2), A/Mississippi/1/85 (H3N2) and A/PR/8/34 (H1N1) differed in the virulence: the ratio of plaque forming units (pfu) needed for 1LD<sub>50</sub> of these viruses for mice was 1215:77:1 (A/Dunedin/4/73: A/Mississippi/1/85: A/PR/8/34). We followed the spread of infectious virus and vRNA into various organs of infected mice. After i.n. infection with 1/2 LD<sub>50</sub> of each virus, infectious virus was detected in lungs, heart and thymus using the rapid culture method and using RT-PCR. Viral RNA was also found in the liver and spleen of mice infected with all examined viruses, and in the kidneys after infection with viruses A/Dunedin/4/73 and A/PR/8/34. In the brain, however, viral RNA was found only after infection with the virus A/Dunedin/4/73, the one with the lowest virulence. To find the route of virus spread outside the lungs, we searched the

presence of infectious virus as well as virus RNA also in blood. Viral RNA was detected in blood after infection with 2 viruses of lower virulence (i.e. A/Dunedin/4/73 and A/Mississippi/1/85). However, no infectious virus was found in the blood, even if the mice were infected with the higher dose (5LD<sub>50</sub>) of virus. The reason for the inability to detect infectious virus in blood could be its very low concentration, below the limit of detection of infectious virus by cultivation. The presence of vRNA in blood did not confirm, but indicated that viruses can be spread to organs outside the respiratory tract probably by the blood route and so cause transient viremia in infected mice in early intervals after infection (12 hours post infection-4. day post infection). To confirm this hypothesis, experiments on the transfer of infectious virus by blood are ongoing.

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1-012

### Fluorescence-tagged influenza virus for tracking of infection cycle

*Jang, Yo Han; Shin, W.J.; HLee, K.K.; Seong, B.L.*

*Department of Biotechnology, College of Engineering, Yonsei University, Republic of Korea*

Single-virus tracking in living cells provides us with the means to visualize the journey of a virus through the infection cycle. The tracking requires a real-time imaging technique that uses fluorescence microscopy to monitor individual virus particles or viral components in live cells.

To successfully track the virus particle, therefore, the virus and cellular structures of interest must be fluorescently labelled. In combination with the fluorescence microscopic technique and resolution, enough to detect single viruses, it may be possible to monitor and dissect each step of virus infection in real-time. Recent advances in reverse genetics have allowed us to manipulate the influenza virus genome to construct engineered viruses.

Through this technical platform, viruses carrying domains that interact with fluorescent dyes could be generated. Thus, hemagglutinin (HA), the major surface protein of the influenza virus, was modified to carry a fluorescent dye interacting domain consisting of an alpha-helical tetracysteine peptide. The virus will be useful for following the fate of individual virus particles and monitoring dynamic interactions between viruses and cellular structures.

## POSTER PRESENTATIONS

1-013

### Virus isolation and species-dependant selection pressure

**Wahlgren, John**<sup>1</sup>; Falk, K.I.<sup>2</sup>; Olsson, G.<sup>2</sup>; Olsen, B.<sup>3</sup>; Lundkvist, Å.<sup>2</sup>

<sup>1</sup>Karolinska Institutet, Sweden; <sup>2</sup>Swedish Institute for Infectious Disease Control, Sweden; <sup>3</sup>Uppsala University, Sweden

Standard procedure for isolation of influenza A viruses is inoculation of embryonated chicken eggs. However, this procedure is known to introduce mutations in the hemagglutinin gene when applied to human adapted influenza viruses. As an increase in mutation frequency has been shown after low pathogenic viruses have been transferred from their natural host to domesticated poultry, we designed a procedure to investigate isolation on an embryonated egg from the sampled species. The HA gene of two viruses, one H4 and one H6 was sequenced from the raw sample. Subsequently, the viruses were then passaged 6 times in the amniotic and allantoic compartments of embryonated mallard eggs. The same virus was then passaged in the same manner in embryonated chicken eggs. Material from each passaged virus will be sequenced targeting the HA gene, and results regarding selection will be presented at the conference.

1-014

### Sialic acid binding of influenza hemagglutinin protein

**Barclay, Wendy**<sup>1</sup>; Shelton, H.<sup>2</sup>; Ayora-Talavera, G.<sup>1</sup>; Jones, I.<sup>2</sup>; Hennessey, M.<sup>3</sup>; Pickles, R.<sup>3</sup>

<sup>1</sup>Imperial College London, UK; <sup>2</sup>University of Reading, UK; <sup>3</sup>University of North Carolina at Chapel Hill, USA

Influenza mediates entry to host cells through interactions between the envelope protein hemagglutinin (HA) and host cell sialic acid moieties. It is generally accepted that avian influenza has a preference for terminal  $\alpha$ 2-3 sialic acid while the human viruses prefer  $\alpha$ 2-6 sialic acid. The expression of the sialic acid moieties can be revealed using lectins and has been shown to vary between cell types and tissues. Here we show that recombinant HA proteins can be used as biologically relevant probes of cell susceptibility in human, ferret, pig and chicken tissues. The pattern of HA binding correlates with susceptibility to virus infection and demonstrates a clear difference in the tropism between human and avian subtypes of influenza A virus. We have mapped the binding of H5 protein from the A/Vietnam/1194/04 strain to ciliated cells in the human airway. Various mutations in H5 HA reported in the literature to switch receptor preference have been assessed in the context of real tissue sections.

1-015

### Induction of inflammatory cytokines in human lung epithelial cells by avian influenza A H7N3

**Chan, Paul**

The Chinese University of Hong Kong, Hong Kong

**Introduction:** Avian influenza A viruses pose the threat of initiating new influenza pandemics. In addition to the current focus on H5N1, other subtypes also have a potential to emerge as the next pandemic virus. For instance, avian-to-human transmission of the H7 subtype occurred during the course of poultry outbreaks in the Netherlands in 2003, and in Canada in 2004, which resulted in mild to severe illnesses in many people working closely with the infected poultry. Viruses belonging to the antigenic subtypes H5 and H7 can be highly pathogenic to humans. In one of the fatal human infections with H7 in 2004, it was reported that acute respiratory distress syndrome and multiple organ dysfunction associated with severe bronchointerstitial pneumonia were observed. Moreover, these syndromes were also frequently detected in the fatal human H5 infections, which were found to be the consequence of hypercytokinemia. The biological basis accounting for the severity of H7 infection in humans remains unknown. We have therefore investigated the profiles of cytokine/chemokine gene expressions, as well as the receptor gene expression induced by avian influenza virus subtype H7 in human lung epithelial cells.

**Methods:** Expression temporal profiles of cytokines/chemokines and their receptor genes in human lung epithelial (NCI-H292) cells following the inoculation of avian influenza A/Canada/504/2004 (H7N3/04) at m.o.i. of 0.1, 1, 10 were monitored by quantitative real-time RT-PCR.

**Results:** An early-phase (3-6hr) induction of several inflammatory cytokines/chemokines: TNF-alpha, IL-6, IL-8, IP-10, CCL-3, CCL5, CCL-8, CCL-20, CXCL-1, CXCL-9, SDF-1, IL-1-beta and IL-10; together with a significant increase in the cytokine/chemokine receptors CCR2 and CXCR4 were seen. The degree of cytokine/chemokine induction was proportional to the concentration of viral inoculum. During this early phase of infection, CCL-3, CCL-5(RANTES), CXCL-9, and IP-10 demonstrated the most prominent response. During the late phase of infection, cytokines/chemokines: IL-6, IL-10, CCL-3, CCL-5(RANTES), CCL-20, CXCL-9, IP-10, IFN-gamma and TNF-alpha were highly expressed (10-1700 folds), together with CXCR5 receptors. The degree of cytokine/chemokine inductions (except CXCL-9 and IP-10) was also proportional to the concentration of viral inoculum. This cluster of induced cytokines/chemokines during the late phase of infection may account for the phenomena of hypercytokinemia that mediate the subsequent pathology.

**Conclusion:** The NCI-H292 cells can support avian H7N3 replication and serve as a model for studying the host response to viral infection. These human lung cells respond to H7N3 infection by an

induction of different inflammatory cytokine/chemokine as well as chemokine-receptor gene expressions, particularly IP-10 and CCL-5(RANTES). These could be potential therapeutic targets.

1-017

### Pathogenicity and transmission studies of influenza A (H2N2) viruses in ferrets

**Pappas, C.<sup>1</sup>**; Van Hoeven, N.<sup>1</sup>; Viswanathan, K.<sup>2</sup>; Chandrasekaran, A.<sup>2</sup>; Sasisekharan, R.<sup>2</sup>; Katz, J.M.<sup>1</sup>; Tumpey, T.M.<sup>1</sup>

<sup>1</sup>Immunology and Pathogenesis Branch, Influenza Division, Centers for Disease Control and Prevention, USA; <sup>2</sup>Department of Biological Engineering, Massachusetts Institute of Technology, USA

The 1957 pandemic was caused by the emergence and spread of the influenza A H2N2 subtype virus. Although these viruses have disappeared from the human population, viruses within this subtype can still be isolated from avian and swine species. In these studies, we used the ferret model that parallels the efficient transmission of H3N2 human viruses and the poor transmission of avian influenza viruses. We assessed two H2N2 viruses with different receptor binding specificities for their ability to undergo efficient respiratory droplet transmission (RDT) between ferrets. The H2N2 (A/Albany/6/58) virus, possessing alpha 2,6 sialic acid ( $\alpha$ 2,6 SA) binding preference, was able to replicate to high titers in the nasal turbinates of the inoculated ferrets and was efficiently transmitted by RDT as well as by direct contact transmission. Clinical signs of infection were similar to seasonal H3 and H1 influenza virus strains and ferrets recovered fully from intranasal inoculation. Conversely, an H2N2 (A/El Salvador/2/57) virus, possessing  $\alpha$ 2,3 SA binding preference, also replicated well among inoculated animals, but it failed to transmit efficiently as evidenced by the paucity of virus shedding and seroconversion among the contact ferrets. Additional work is underway to further address receptor binding, the initial event in influenza virus infection, as a major determinant of virus transmission efficiency of H2N2 influenza viruses.

1-018

### Sequence variation in influenza A virus NS1 protein that affects control of interferon response in avian cells

**Barclay, W.S.<sup>3</sup>**; Linton, Sarah<sup>1</sup>; Daly, J.<sup>2</sup>; McCrae, S.<sup>2</sup>; Elton, D.<sup>2</sup>;

<sup>1</sup>Imperial College London, UK; <sup>2</sup>Animal Health Trust, UK; <sup>3</sup>Imperial College London, UK

The influenza A virus NS1 protein modulates the innate immune response by blocking induction and effects of interferon- $\alpha/\beta$  (reviewed by Haller, Kochs and Weber, 2006). This study aims to elucidate the role of NS1 in the pathogenicity of a set of equine influenza A viruses. Four different strains of H3N8 subtype equine influenza viruses, isolated in the UK between 1989 and 2003 replicated equally well in vivo but varied in levels of interferon- $\alpha/\beta$  and interleukin-6 they induced in infected ponies and this correlated with disease severity (Wattrang *et al.*, 2003; unpublished observations by Janet Daly and Richard Newton). All of the equine NS1 proteins block IFN- $\beta$  induction, IRF-3 nuclear localisation, and expression of transiently induced genes in human A549 cells when expressed exogenously or expressed from a recombinant human virus background. However in a chicken cell line, DF-1, the two equine influenza viruses associated with more severe disease in the horse both induced higher levels of interferon than did a virus associated with milder symptoms. This phenotype mapped to the NS1 gene because exogenous expression of NS1 protein in DF-1 cells was unable to block an induced response. Furthermore recombinant human viruses carrying the RNA segment 8 encoding NS1 from each of the equine strains mimic the cytokine induction phenotype of the wild-type equine influenza viruses. The NS1 proteins from two viruses displaying different pathogenicity phenotype differ at only two amino acid positions. These data illustrate that phenotypes that are determined by interaction between viral NS1 protein and host cell components may depend on the species infected. Thus during a zoonotic event a high virulent phenotype conferred by the NS1 gene may not always be transferred to the new host.

1-020



### Novel siRNA-Chimeric-Rz construct potently interferes with the replication of influenza virus

**Khanna, M.<sup>1</sup>;** Vyas, R.<sup>1</sup>; Sood, V.<sup>2</sup>; Vijayan, V.K.<sup>1</sup>; Akhil, C.<sup>3</sup>

<sup>1</sup>V P Chest Institute, University of Delhi, Delhi, India; <sup>2</sup>National Institute of Immunology, India; <sup>3</sup>National Institute of Immunology, JNU Campus, Aruna Asaf Ali Marg, New Delhi, India

A multi-target approach is needed for effective gene silencing for RNA viruses that combines more than one antiviral approach. Towards this end, we designed a wild type (wt) chimeric construct that consisted of small hairpin siRNA joined by a short intracellular cleavable linker to a known hammerhead ribozyme, both targeted against the M1 genome segment of the influenza virus. When this wt chimeric RNA construct was introduced into a mammalian cell line, along with the M1 substrate encoding DNA, very significant (67%) intracellular down regulation in the levels of target RNA was observed. When the siRNA portion of this chimeric construct was mutated, keeping the Rz region unchanged, it caused only 33% intracellular reduction. On the contrary, when only the Rz was made catalytically inactive, keeping the siRNA component unchanged, about 20% reduction in the target M1-specific RNA was observed. This wt chimeric construct showed impressive (>80%) protection against virus challenge, on the other hand, the selectively disabled mutant constructs were less effective. Thus, in this proof-of-concept study we show that varying levels of protection against virus challenge was observed with novel mutant versions of the chimeric constructs.

1-021

### Mammalian pulmonary endothelial cells support productive replication of highly pathogenic avian influenza A H5N1 viruses

**Zeng, H.<sup>1</sup>;** Stevens, T.S.<sup>2</sup>; Katz, J.M.<sup>1</sup>; Balczon, R.<sup>2</sup>; Tumpey, T.M.<sup>1</sup>

<sup>1</sup>Immunology and Pathogenesis Branch, Influenza Division, Centers for Disease Control and Prevention, Atlanta, Georgia, USA; <sup>2</sup>Department of Pharmacology, and Center for Lung Biology, University of South Alabama, Mobile, Alabama, USA

Highly pathogenic avian influenza (HPAI) A H5N1 viruses have caused not only devastating outbreaks in domestic poultry but also sporadic human infections with a high fatality rate. Respiratory failure with acute respiratory distress syndrome has been the major complication among hospitalized patients. The nature of the interactions between the H5N1 influenza virus and the host pulmonary vasculature is largely unknown. To better understand the pathogenic mechanisms of pulmonary endothelial injury associated with H5N1 virus infections, we utilized four pulmonary endothelial cell types, including primary human lung microvascular endothelial cells (HMVEC-LBI), a human lung microvascular endothelial cell line (HULEC), primary rat pulmonary microvascular endothelial cells (PMVEC) and primary rat pulmonary arterial endothelial cells (PAEC). Evaluation of these cells for the presence of the known sialic acid (SA) receptors preferred by influenza viruses demonstrated that alpha 2,3 SA configuration (preferred by avian influenza viruses) was preferentially found on all endothelial cell types tested. To test the permissiveness of pulmonary endothelial cells to influenza virus infection, the cells were seeded onto chamber slides and infected with selected human H1N1, H3N2, and HPAI H5N1 viruses at MOI of 1. Nucleoprotein (NP) staining, representing a suitable marker of ongoing viral replication, demonstrated a higher percentage rate of H5N1 virus-infected cells compared to endothelial cells infected with human H1N1 and H3N2 viruses. By comparing the replication kinetics of these influenza viruses in endothelial cells, we observed that these cells only support productive replication of H5N1 viruses. Furthermore, studies on endothelial cells seeded on transwells suggested that H5N1 viruses preferentially enter through and are released from the apical surface of polarized human endothelial monolayers. Further analysis of the interaction between the H5N1 virus and pulmonary endothelial cells, which represent targets of viral infection by this subtype, may shed light on the virulence and pathogenesis of the H5N1 virus in humans.

## 2 CLINICAL IMPACT AND DIAGNOSTIC APPROACHES

2-001

### How do influenza rapid tests improve the management of influenza in children in primary care?

**Cohen, C.R.<sup>1</sup>; Levy, L.C.<sup>2</sup>; Caron, C.F.M.<sup>3</sup>; de La Rocque, F.L.R.<sup>2</sup>; Languet, J.L.<sup>3</sup>**

<sup>1</sup>INFOVAC, ACTIV, CHI Créteil, France; <sup>2</sup>ACTIV, France; <sup>3</sup>AFPA, France

**Background:** Previous studies showed that clinical diagnosis of influenza based on symptoms lacks accuracy in children. The aim of this study is to evaluate the impact of the influenza rapid test (IRT) in the management of influenza-like illnesses in children in primary care.

**Methods:** During the 2007-2008 influenza season, an observational prospective, multicenter study was carried out in France. Patients were enrolled after national influenza activity first increased above baseline levels according to the French influenza surveillance network GROG (Groupes Régionaux d'Observation de la Grippe). Clearview influenza A&B tests® were used for qualitative detection of influenza A and B virus antigen directly from nasal swabs.

The impact of IRT on disease management was evaluated by antiviral and antibiotic prescription, according to risk factors, influenza vaccination status and time after onset of symptoms (fever, myalgia, headache, shivers, asthenia, cough, nasal discharge...). Data centralization is still in process but final analysis will be available in September.

**Results:** This interim analysis reports 50% of data collected up-to-date. In a 17-week period [week 47 - week 11], during influenza activity, 242 pediatricians and general practitioners included 5463 children with clinical symptoms suggesting influenza. The vast majority of IRT were performed between week 2 to week 4 (52.4%). The mean age of children was  $4.6 \pm 3.2$  years. Children between 1 and 5 years old accounted for 66.2% of cases. For 72.8% of patients, the onset of symptoms was less than 48h ( $32.4\% < 24h$ ,  $12.7\% < 12h$ ). High risk children according to French recommendations for influenza vaccine were 5.5%. Among those, only 30.5% were vaccinated. Inaugural acute otitis media and/or pneumonia were diagnosed for 12.5% of children (n=653).

IRTs were positive in 47.1%, negative in 51.5% and not assessable in 1.4% of cases. Diagnosis of influenza was confirmed by IRT in 43.8% of children under 5 years old and 57.2% in children more than 6 years old ( $p < 0.0001$ ). Whatever the population is (i.e. overall population, at risk children or children with acute otitis media and/or pneumonia), according to IRT results, antiviral and antibiotic use is more targeted, i.e. practically no antiviral use and significantly more antibiotic use when IRT negative compared to positive IRT.

	Antiviral prescription	Antibiotic prescription	Parental absenteeism related to child care
Overall population			
Positive IRT	27.4% (678/2473)	10.8% (267/2466)	34.3% (842/2457)
Negative IRT	1.5% (39/2685)	23.9% (643/2694)	24.3% (643/2648)
	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
High risk children			
Positive IRT	29.5% (39/132)	20% (27/135)	35% (48/137)
Negative IRT	2.1% (3/145)	35.2% (51/145)	25.4% (35/138)
	$p < 0.0001$	$p = 0.004$	$p = 0.08$
Children with acute otitis media and/or pneumonia			
Positive IRT	15.5% (36/233)	81.7% (192/235)	45.3% (106/234)
Negative IRT	0.3% (1/380)	90.7% (350/386)	29.4% (111/378)
	$p < 0.0001$	$p = 0.001$	$p < 0.0001$

**Conclusion:** The interim results of this study confirm that IRT can improve the diagnosis of influenza in children, even among children older than 6 years and even at the peak of influenza activity. More targeted influenza management with antivirals and antibiotics occurs when IRT results are available, in all patients groups (i.e. overall population, at risk children or children with acute otitis media and/or pneumonia). This study also shows that influenza vaccination among high risk children still needs to be improved.

2-002

### Comparative investigations of microneutralization and hemagglutination inhibition tests in evaluation of immunogenicity of inactivated influenza H5 vaccine

**Sominina, A.A.<sup>1</sup>; Krivitskaya, V.Z.<sup>1</sup>; Mironov, A.N.<sup>2</sup>; Voytsekhovskaya, E.M.<sup>1</sup>; Vasileva, A.A.<sup>1</sup>; Tretjakova, N.V.<sup>1</sup>; Kiselev, O.I.<sup>1</sup>**

<sup>1</sup>Research Institute of Influenza (RII), St. Petersburg, Russian Federation;

<sup>2</sup>Scientific-Production Association "Microgen", Russian Federation

The emergence of avian A(H5N1) virus in humans and its widespread distribution highlights the need for carrying out seroepidemiological studies to determine the mode of virus transmission and risk factors associated with infection. Therefore, there is a need for sensitive, specific and reliable serological testing. Routine hemagglutination inhibition (HI) testing using chicken or turkey erythrocytes appeared to be insensitive in detection of antibody response to avian hemagglutinin after

vaccination or infection, even in the presence of virus neutralizing antibodies. The modified design of the microneutralization assay (MN) to increase the biosafety of procedures in determining antibodies to influenza A(H5N1) virus and to simplify the test was developed. Influenza vaccine strain NIBRG-14 obtained by reverse genetic technology and containing HA and NA from the A/Viet Nam/1194/04 virus kindly provided by Dr. John Wood (NIBSC, UK) was used in the MN and HI assay. Such an approach allowed for conducting an investigation of sera with a live H5 virus in BSL 2 conditions. Briefly, the tested sera were heated at 56°C for 30 min and diluted from 1:10 up to 1:1280 and were mixed with equal volumes of 100 TCID<sub>50</sub> virus. After incubation for 1 hour at 37°C they were introduced into the wells of microplates with a monolayer of MDCK cells. After 48 h incubation at 37°C, the cells were fixed by cold acetone and an ELISA was carried out. Direct detection of virus antigens due to the application of peroxidase (PO) conjugate of monoclonal antibodies (MAbs) 4H1 to the conservative site in NP of the influenza A virus developed at RII (T.R. Tsareva *et al.*, 2007) was used. Thus, the need for the second ELISA stage (PO conjugate of antibody to mouse IgG) was excluded. Non-carcinogenic tetramethylbenzidine instead of orthophenyldiamine was applied as a chromogen. The specificity of MN using MAb 4H1 PO conjugate had been evaluated previously. No antibodies (Abs) to influenza A(H5N1) virus were detected in sera obtained from 10 volunteers vaccinated by season trivalent influenza vaccine "Grippol", 13 sera of ILI reconvalescents or 33 sera taken from healthy poultry workers (titer of Abs was lower than 1:10). At the second step, immunogenicity of the H5 vaccines (NIBRG-14 strain) was evaluated in an investigation of 324 sera from 108 volunteers immunized with inactivated influenza A(H5N1) vaccine at RII. With dependence on vaccine formulation prepared at "Microgen" Enterprises, namely the content of virus hemagglutinin, adjuvant or immunomodulator (V.V. Zverev *et al.*, 2007), geometric mean titers (GMT) of Abs varied within 1:6.5 - 1:14.7 after the first vaccination and increased up to 1:23.1 - 1:58.9 after the second vaccination. Abs titers 1:40 and higher in MN (presumably assessed as protective) were registered in 18.5% and 62.9% of volunteers after the first and second vaccination, respectively. The Abs titer increases accounted for 4.6 - 11.8 according to the formulation of the vaccine. Comparative investigations of the same sera in HI testing (sera were treated by RDE, human erythrocytes of o group, Rh+ were used) showed that antibody titers were higher in MN than in the HI test by 1.2-2.2 times in different groups of vaccinated volunteers. The main differences between the two tests results could be explained by the higher MN sensitivity in comparison with the HI test. Comparative investigations of MN and HI tests efficacy were continued to estimate two variants of influenza H5-vaccine which differed in hemagglutinin content (the second phase of trials). Horse erythrocytes (HoE) were used in the HI test at this stage of the investigation as GMTs in postvaccination sera with HoE were higher than by use of human erythrocytes. GMTs of Abs determined in MN after twofold immunization with vaccine-1

(55 volunteers) and vaccine-2 (54 volunteers) were 16.4% and 28.7% higher than in the HI test using HoE and recorded 1: 43.1 and 1:50.9. Other indices were also higher in MN as compared with the HI test: rate of seroconversions after immunization with vaccines -1 and 2 - at 10.9% and 20.4%, protective titers of Abs - 0% and 9.3%, ratio of Abs titers increase - 1.6 and 2.3 times higher, respectively. Furthermore, false positive results (detection of Abs in sera of volunteers before vaccination in titer 1:20 or higher) were more frequent in HI than in MN (10% and 1.7% correspondingly). It was revealed additionally that results of HI in detection of H5-specific Abs in sera of vaccinated people depended on individual properties of the erythrocyte donor. Thus, results of these investigations indicated higher sensitivity and specificity of the MN assay as compared to the HI test and expediency of its application in evaluation of new influenza A(H5N1) vaccines.

2-003

#### The design of alternative immune assays to determine a protective immune response against influenza, and application thereof in vaccine trials

Gijzen, Karlijn<sup>1</sup>; Pronk, I.<sup>1</sup>; Liu, W.M.<sup>1</sup>; Oftung, F.<sup>2</sup>; Korsvold, E.G.<sup>2</sup>; Visontai, I.<sup>3</sup>; Tutto, A.<sup>3</sup>; Jankovic, I.<sup>3</sup>; McElhaney, J.<sup>4</sup>; Lemire, Y.<sup>4</sup>; Soethout, E.<sup>1</sup>

<sup>1</sup>Netherlands Vaccine Institute, Netherlands; <sup>2</sup>Norwegian Institute of Public Health, Norway; <sup>3</sup>National Centre for Epidemiology, Hungary; <sup>4</sup>University of Connecticut Health Center, USA

**Introduction:** A main objective of the EU-funded FLUSECURE program is to predict vaccine efficacy by developing new correlates of protection (COPs). The classical method in predicting efficacy of influenza vaccines is by measuring the antibody response to the HA antigen which is however inadequate in especially elderly people. In addition, the often used hemagglutination inhibition (HI) assay may yield inconsistent results. Therefore, the development of new predictive parameters is essential. It is crucial that new parameters are validated before application by multiple laboratories. This will ensure the standardized and reliable determination of immune responses. Complete protection against an influenza infection may require both the humoral and cellular immune response. Since the standard humoral assays are not sufficient as markers for protection, we applied new techniques based on the cellular and humoral response against influenza.

**Results:** We chose to validate novel markers of the cellular immune response. In addition, the neuraminidase inhibition assay was applied to determine the humoral immune response against neuraminidase. The cellular assays comprise stimulation of PBMC

with live influenza virus and subsequent detection of granzyme B and cytokines that is produced. The assays were validated for general use in multiple laboratories from different countries. Validation results showed acceptable variation and robustness. Subsequently, the validated assays were tested in two human clinical trials in which healthy individuals were vaccinated with either split vaccine (in the Netherlands) or whole virus vaccine (in Hungary). Preliminary results show detectable granzyme B responses in vaccinated individuals. Furthermore, an increase in neuraminidase inhibition was observed after vaccination with the whole virus vaccine. Moreover, the outcome of the cellular and humoral assays will be correlated to protection, since the participants of the split vaccine trial were monitored for infection during the influenza season.

**Conclusion:** Identification of new COPs for determination efficacy of influenza vaccines is essential since assessment of HI and SRH titers such as current COPs are insufficiently indicative of protection. Promising options are assays based on cellular immune response and the NI assay. We determined standard protocols to analyze the cellular immune response which is a prerequisite for their general application. In addition, the assays were applied in unique human clinical trials indicating their usefulness for vaccine development.

2-004

#### Molecular detection and identification of human and avian influenza viruses by European National Influenza Centres: results of two subsequent external quality assessments

Meijer, A.<sup>1</sup>; MacKay, W.G.<sup>2</sup>; van Loon, A.M.<sup>2</sup>; Niedrig, M.<sup>3</sup>; Lina, B.<sup>4</sup>; Niesters, H.G.M.<sup>2</sup>

<sup>1</sup>EISS Coordination Centre, Utrecht; National Inst. for Public Health and the Environment, Bilthoven, Netherlands; <sup>2</sup>Quality Control for Molecular Diagnostics (QCMD), Glasgow, UK; <sup>3</sup>Robert Koch Institute, Berlin, Germany; <sup>4</sup>National Influenza Centre for South France, Lyon, France

**Background:** In Europe, influenza is monitored by the European Influenza Surveillance Scheme (EISS), integrating epidemiological and virological surveillance data. Molecular detection techniques are increasingly important for rapid diagnosis, especially with regard to pandemic preparedness. To assess the performance of EISS affiliated National Influenza Centres (NICs) in molecular detection and identification of human and avian influenza viruses, EISS jointly with Quality Control for Molecular Diagnostics (QCMD), carried out two subsequent external quality assessments.

**Methods:** In 2006 and 2007, almost identical (13/14 panel members) panels composed of inactivated influenza viruses A(H1N2), A(H7N3) and B, dilution series of A(H3N2) and A(H5N1), and negative specimens were distributed to the NICs, which were

given six weeks to return results. Results with the 13 identical panel members of the 28 NICs (23 countries) that returned results for both studies were compared.

**Results:** The median percentage correct results by NIC increased from 68% to 82% for detection and typing and from 67% to 87% for subtyping of detected type A viruses. However, the proportion of NICs reporting false positives remained identical: 5 of 28 in both years, with 6 false positives in 2006 and 7 in 2007. With a virus containing a panel member, the median percentage correct results increased from 68% to 82% for detection and typing and from 64% to 83% for subtyping of detected type A viruses. The main reason for incorrect typing and subtyping was false negatives being proportional to virus concentration. Identification of another subtype was the second main reason for incorrect subtyping.

**Conclusion:** Although detection and identification have improved, a high proportion of false positives was still reported. Therefore, further improvement is needed to assure rapid correct detection and identification, especially in cases where influenza virus infection is life threatening e.g. with A(H5N1) virus.

2-005

#### Application of a quantitative real-time RT-PCR assay for influenza viruses in the analysis of viral load in clinical material

Ellis, J.<sup>1</sup>; Democratis, J.<sup>2</sup>; Andrews, N.<sup>3</sup>; Zambon, M.<sup>1</sup>

<sup>1</sup>Influenza Laboratory, Virus Reference Department, Centre for Infections, Health Protection Agency, UK; <sup>2</sup>University Hospitals Leicester NHS Trust, Infirmary Square, Leicester, UK; <sup>3</sup>Statistics Unit, Statistics, Modelling and Bioinformatics Department, Centre for Infections, Health Protection Agency, UK

Quantitative real-time reverse transcription PCR (qRT-PCR) has become the gold standard for detection and quantitation of RNA targets, and is being utilised increasingly in clinical diagnostic assays. A quantitative, multiplex, real-time RT-PCR assay was developed for the detection of influenza A H<sub>1</sub>, H<sub>3</sub> and influenza B viruses, and was extensively validated prior to use on clinical material. The usefulness of the qRT-PCR assay for analysing influenza virus shedding in surveillance clinical specimens, including specimens from influenza vaccinated and unvaccinated individuals, respiratory samples collected following experimental infection, and in samples containing either sensitive or drug resistant influenza viruses, was investigated.

The qRT-PCR assay was applied to the analysis of viral load in respiratory surveillance samples collected during several influenza seasons in the United Kingdom. The results were analysed for correlations between viral load and factors including age of the patient, vaccination status and the period (days) from onset to

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sample collection. Statistical analysis was applied to determine if the differences seen in viral load between groups were significant. The results demonstrated a significant relationship between increasing age and decreasing viral load.

The viral load in respiratory samples collected following experimental infection with A/Panama/2007/99 H3N2 virus was also analysed by qRT-PCR. The pattern of virus shedding was determined by daily sample collection, for 7 days following infection, and analysis by qRT-PCR. The qRT-PCR allowed evaluation of the variability in virus shedding in different sample types, and the temporal pattern of shedding.

Lastly, the qRT-PCR assay was also applied to the study into the shedding and persistence of drug resistant viruses in influenza infections in humans. The results of our investigations demonstrate that qRT-PCR is useful for beginning to analyse virus shedding versus transmissibility and duration of symptoms in infections with influenza viruses

2-006

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### Quest for causes of inefficient hemagglutination of recent A(H3N2) influenza viruses

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**Meijer, A.;** Tjeerdsma-de Bruin, G.; Overduin, P.; van der Lubben, I.M.; Jonges, M.

*National Institute for Public Health and the Environment, Bilthoven, Netherlands*

**Background:** During the surveillance of influenza in the 2005/2006 and 2006/2007 winter seasons in the Netherlands, an increasing proportion of influenza A(H3N2) virus isolates hemagglutinated poorly or not at all the routinely used turkey red blood cells (RBCs) for the hemagglutination (HA) and hemagglutination inhibition assays. The proportion poorly hemagglutinating A(H3N2) viruses increased from 32% in 2005/2006 to 75% in 2006/2007. This phenomenon delayed the timely correct identification and antigenic characterization of these viruses and possible causes were investigated.

**Methods:** Firstly, the possible improvement of HA by the use of RBCs from different species was investigated. Secondly, the amino acid (aa) changes in the receptor-binding site of selected A(H3N2) influenza viruses were systematically monitored during serial passage of primary isolates on tertiary monkey kidney (tMK) and MDCK-I cells.

**Results:** RBCs from guinea pigs and humans (with RBC type O, A, B and AB) did not significantly improve the HA of the poorly hemagglutinating A(H3N2) viruses. Some improvement of HA of a few of these viruses was seen after growing large batches for other purposes. Therefore, four A(H3N2) virus strains with a low or absent HA titer and with a slightly different hemagglutinin

nucleotide sequence were selected for further investigation of aa changes in the receptor-binding site during serial passage of these viruses.

For three of the viruses, improvement of HA was accompanied by an aa change from leucine to proline at position 194 in the receptor-binding site of the hemagglutinin. During six passages on tMK cells, the viruses with proline gradually replaced viruses with leucine for up to 100%. However, during six passages on MDCK-I cells, only one virus maintained the proline whilst for the other two viruses the proportion viruses with proline peaked at passage three or four and reverted almost completely to leucine by passage six, accompanied by a reduction in hemagglutination capacity.

Although other A(H3N2) viruses from both seasons also had leucine at position 194, they had normal HA titers with turkey RBC. As the nucleotide sequence of the hemagglutinin gene of the four selected viruses was slightly different, aa at other critical positions likely play a role in: 1) whether the aa at position 194 is critical for HA capacity, and 2) whether the virus has the capacity to accept and maintain the point mutation resulting in the observed aa substitution at position 194. In a similar way, the introduction of leucine at position 194 together with certain types of aa at other critical positions in the hemagglutinin previously played a concerted role in the lost ability of A(H3N2) viruses to agglutinate chicken RBC.

**Conclusions:** For cell culture diagnosis of influenza, the correct identification and characterization of A(H3N2) influenza viruses causing a cytopathological effect (CPE) in combination with poor HA might be improved by passage of these viruses an additional three or four times on tMK or MDCK-I cells, thereby causing an aa change from leucine to proline at position 194 in the receptor binding site of the hemagglutinin and increased HA. Further research is needed to investigate what the other critical aa substitutions for reduced HA are and whether these substitutions might result in changed virulence of the virus.

2-007

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### Influenza HA and NA-pseudotyped retroviral vectors: applications to pandemic vaccine evaluation, sero-surveillance and antiviral drug screening

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**Temperton, Nigel**

*University College London, UK*

The continuous rapid evolution of H5N1 influenza viruses has major implications for the sensitivity of serological assays and can limit the efficacy of avian and pandemic human vaccines and the susceptibility of these viruses to anti-virals. Retroviral pseudotypes bearing HA and NA envelope glycoproteins are

ideally placed to address these problems and can be used for;

1. Sensitive, high-throughput, low-containment cell-based assays for neutralizing antibodies against influenza H5N1 HA. It is straightforward to update this HA neutralization assay to measure responses against newly emerging HA antigenic drift variants. Upon availability of the HA sequence of the emergent H5N1 virus, the HA can be synthesized and retroviral pseudotypes prepared for use in neutralization assays. These assays can be used to address the cross-clade neutralizing potential of pandemic human vaccines and immuno-therapeutics (monoclonal antibodies etc.), and for sero-surveillance studies in new outbreak locations.
2. Sensitive assays for neutralizing antibodies against NA.
3. Sensitive assays for the evaluation of anti-HA and anti-NA drugs and for the study of drug resistance.

2-008

#### Neutralizing monoclonal antibodies to different clades of H5N1 influenza A viruses

**Oh, Sawyin<sup>1</sup>**; Selleck, P.<sup>2</sup>; Temperton, N.J.<sup>3</sup>; Chan, P.<sup>4</sup>; Manavis, J.<sup>1</sup>; Higgins, G.<sup>5</sup>; Burrell, C.J.<sup>6</sup>; Kok, T.W.<sup>7</sup>

<sup>1</sup>Institute of Medical and Veterinary Science, Adelaide, Australia; <sup>2</sup>AAHL, CSIRO, Geelong, VIC, Australia; <sup>3</sup>University College London, London, UK; <sup>4</sup>Prince of Wales Hospital, Shatin, Hong Kong, China; <sup>5</sup>Institute of Medical and Veterinary Science, Australia; <sup>6</sup>Institute of Medical and Veterinary Science/University of Adelaide, Adelaide, Australia; <sup>7</sup>Institute of Medical and Veterinary Science/University of Adelaide, Adelaide, Australia

The viral hemagglutinin (HA) surface glycoprotein is the primary target of neutralizing antibodies. Monoclonal antibodies (mAbs) were generated by fusion of SP2/o myeloma cells and splenocytes from mice immunized with  $\gamma$ -irradiated, sucrose density gradient purified influenza A/Chicken/Vietnam/8/2004 H5N1 virus. Positive hybridomas were identified by ELISA using the homologous antigen. We have established four HA-specific IgG1 mAbs and three of these mAbs (1C1, 2C3B, 2D2) exhibited high antibody neutralizing activity to H5N1 strains from clades 1, 2 and 3 (www.who.int). Of the six H5N1 strains tested, mAb 1C1 showed the strongest neutralizing activity with an IC<sub>50</sub> titre of 51200 to a human strain, A/Vietnam/1203/2004 determined using the murine leukemia virus (MLV) pseudotype assay. The relative neutralizing activities of the mAbs were confirmed by live virus microneutralization. In hemagglutination-inhibition (HAi) tests using the homologous antigen, only mAb 1C1 gave a titre of 128. ELISA titrations of the four mAbs including the non neutralising mAb1F7 showed no reactivity to 15 other influenza A subtypes over a range of antibody concentrations. mAbs 1C1 and 1F7 recognised linear epitopes on HA reacting with

a band at ca 50 kDa on western blots. All four mAbs reacted strongly to the homologous antigen by immunohistochemical staining and on MDCK cells infected with the human strains, A/Hong Kong/483/97 or A/Thailand/1(KAN-1)/04 H5N1 by immunofluorescence. These results suggest that the mAbs are useful reagents for the construction of an H5N1 specific rapid point of care biosensor<sup>a</sup> and other immuno-based diagnostic tools or further development for potential therapeutic use.

<sup>a</sup> Oh S.Y., Cornell B., Smith D., Higgins G., Burrell C.J., Kok T.W. *Biosensors & Bioelectronics* 2008; 23: 1161-1165.

2-009

#### Lyophilized Real-Time One-Step RT-PCR tests for detection of avian influenza A/H5/N1/H7 viral isolates

**Schumaker, Michael**; Wong, L.; Brzoska, P.; Wong, A.; Furtado, M.; Petrauskene, O.

Applied Biosystems, USA

In response to continued worldwide outbreaks of avian influenza, we have developed highly sensitive and specific real-time one-step RT-PCR tests for detection of influenza A (M gene target), subtypes H5 and H7 (hemagglutinin gene target), and subtype N1 primarily in H5N1 strains (neuraminidase gene target). To simplify testing workflows and storage for public health officials and researchers performing routine testing or targeted surveillance, each test is completely lyophilized (including complete one-step RT-PCR components) into a single, easily dissolvable bead. Each test requires the addition of just RNA and a reconstitution solution for a dry bead. In addition to target primer and probe sets, each lyophilized reaction includes an internal positive control to provide a sensitive means of monitoring the presence of materials that can inhibit nucleic acid amplification. The assays (primer and probe sets) were developed and validated using our automated TaqMan<sup>®</sup> assay design algorithms and pipeline for accurate and efficient detection of target nucleic acid sequences. We utilized these algorithms to generate sets of assays to specifically detect the majority (> 95%) of recently (post-2000) identified influenza isolates. Each assay involves amplification of multiple regions to enable broad strain coverage and increase the probability of detecting evolving viral strains. High inclusivity of the assays was demonstrated by thorough in silico analysis and confirmed using a broad range of avian influenza isolates in validation studies conducted in several influenza testing laboratories globally. The lyophilized tests demonstrated high efficiency and sensitivity (10-100 copies per reaction) for a broad range of influenza A strains when used in combination with Applied Biosystems 7500/7500Fast/7300/7000/7900HT/StepOne<sup>™</sup> Real-Time PCR

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Instruments. Enhanced inclusivity and sensitivity of the tests will increase the probability of detecting newly emerging strains that can result from genetic drift within influenza A and H5N1 and H7 subtypes. Our bioinformatics algorithms and assay designs can be rapidly optimized to include new sequence information as it becomes available for newly emerging, highly pathogenic influenza isolates. For research use only. Not for use in diagnostics. Not for sale in the US.

2-011

### Comparison of haemagglutinin-inhibition using horse erythrocytes and neutralising antibody assays for detection of antibody to influenza A/H5N1 viruses: an international collaborative study to evaluate an international standard for H5 antigen

**Stephenson, Iain**<sup>1</sup>; Newman, R.<sup>2</sup>; Major, D.<sup>2</sup>; Heath, A.<sup>2</sup>; Wood, J.M.<sup>2</sup>; Katz, J.<sup>3</sup>; Zambon, M.C.<sup>4</sup>; Weir, J.<sup>5</sup>; Levandowski, R.<sup>6</sup>

<sup>1</sup>University of Leicester, UK; <sup>2</sup>NIBSC, UK; <sup>3</sup>CDC Influenza Division, USA; <sup>4</sup>Respiratory Virus Lab, HPA, UK; <sup>5</sup>CBER, USA; <sup>6</sup>National Institute for Allergy and Infectious Disease, USA

**Background:** Traditional haemagglutinin-inhibition (HI) tests are used to detect antibody to influenza. Due to the relative insensitivity of conventional HI for some avian influenza antibodies, modified HI assays using horse red blood cells (hHI) and virus neutralization are employed for H5 antibody assays. Difficulties in comparing neutralising and hHI titres include lack of standard protocols, differences in sensitivity and specificity of reagents and assay variability. A comparative study assessing neutralising antibody responses to influenza A/H3N2 demonstrated significant intra and inter-lab variation. However, variability was significantly reduced by the use of a serum standard, and the development of an international standard for H5 has been given high priority. A study was performed to investigate the reproducibility of hHI and neutralising assays for detection of antibody to avian influenza H5N1. The aims were to compare intra and inter-laboratory variation of serum titres of antibody to H5N1 and to evaluate an international standard (IS) in reducing assay variability.

**Methods:** Participants in seventeen laboratories from ten countries were sent three reassortant reverse-genetic H5N1 viruses: NIBRG-14 (A/Vietnam/1194/2004), NIBRG-23 (A/turkey/Turkey/1/2005) and IBCDC-RG5 (A/Anhui/1/2005). A panel of serum samples including 16 coded human and post-infection sheep sera was prepared. A freeze-dried international standard was prepared from sera obtained from vaccinated donors in clinical trials. Participating laboratories were required to supply results from 3 independent hHI and neutralising assays for all

sera against all of the supplied H5N1 viruses.

**Results:** At the time of writing, laboratories are completing the study and are returning data for analysis. Results and findings will be presented.

2-012

### Pyrosequencing of real-time RT-PCR amplicons for rapid sequence confirmation and characterization of highly pathogenic avian influenza A(H5N1) viruses

**Shu, B.**; Deyde, V.M.; Winter, J.; Sheu, T.G.; Gubareva, L.V.; Klimov, A.I.; Lindstrom, S.E.

Centers for Disease Control and Prevention, Atlanta, Georgia, USA

**Background:** Ongoing circulation of highly pathogenic avian influenza A(H5N1) viruses (HPAI) in poultry as well as infrequent infections in humans underline the need for rapid and sensitive diagnostic methods for detection of these viruses. Real-time RT-PCR (rRT-PCR) is currently used in many public health laboratories for diagnostic testing for HPAI virus infections in humans. However, confirmation of rRT-PCR results and further characterization of viruses is often difficult because of problematic issues with the specimens, such as limited volume, low viral load, or poor quality of original clinical samples. In such cases, confirmation of rRT-PCR diagnostic results and generation of genetic data for additional strain characterization may only be achieved by genetic sequencing of rRT-PCR amplicons. Also, sequence confirmation of rRT-PCR amplified DNA products can be used to discriminate A(H5) Eurasian low pathogenic avian influenza viruses (LPAI) as well as rule out false positive results that are due to contamination by positive control RNA. Since fluorescence-based dideoxy-mediated termination methods for nucleotide sequencing are time consuming and labor intensive, we have explored pyrosequencing as an alternative method to increase the throughput of the sequencing analysis of rRT-PCR amplified DNA products.

**Methods:** Pyrosequencing is an alternative method for rapid sequence analysis of short DNA fragments that are similar in length to those amplified by rRT-PCR. In order to allow for post-amplification sequence analysis of the HA gene by pyrosequencing, the CDC rRT-PCR assay for detection of Asian HPAI A(H5N1) viruses (H5a set and H5b set) was modified by biotinylation of one of the two amplification primers.

**Results:** Modification of the rRT-PCR assay by biotinylation of amplification primers did not have any detectable effect on assay performance. High quality sequence data were obtained by standard pyrosequencing of amplified DNA products from all rRT-PCR positive reactions.

**Conclusions:** Real-time RT-PCR assays for detection of HPAI

A(H5N1) viruses were modified to allow for post amplification analysis of DNA products by pyrosequencing. The combined use of rRT-PCR and pyrosequencing technologies makes it possible to rapidly generate confirmatory sequence data that can provide additional genetic information for further characterization and clade specific classification of the HA gene of A(H5N1) viruses. Sequence confirmation of rRT-PCR amplified DNA products can also be used to discriminate between low and highly pathogenic Eurasian avian influenza A(H5) viruses and rule out false positive results that are due to contamination by positive control RNA. Pyrosequencing is a useful method for generating valuable sequence information from rRT-PCR amplified DNA products.

2-013

### How do influenza rapid tests improve the management of influenza in a pediatric emergency care unit?

**Cohen, C.R.<sup>1</sup>;** Levy, C.<sup>2</sup>; Biscardi, S.B.<sup>3</sup>; Angoulvant, F.A.<sup>4</sup>; Lécuyer, A.L.<sup>2</sup>; Mosnier, M.A.<sup>5</sup>

<sup>1</sup>INFOVAC, ACTIV, CHI Créteil, France; <sup>2</sup>ACTIV, France; <sup>3</sup>CHI CRETEIL, France; <sup>4</sup>Robert Debré Hosp, France; <sup>5</sup>GROG, France

**Background:** Previous studies showed that diagnosis of influenza based on symptoms lacks accuracy in children. The aim of this study is to evaluate the impact of the influenza rapid test (IRT) in the management of influenza -illnesses in a pediatric emergency care unit.

**Methods:** During the 2007-2008 influenza season, an observational prospective study was carried out in France. Patients were included after national influenza activity first increased above baseline levels according to the French influenza surveillance network GROG (Groupes Régionaux d'Observation de la Grippe). Clearview influenza A&B tests® were used for qualitative detection of influenza A and B virus antigen directly from nasal swabs.

The impact of IRT on disease management was evaluated by antiviral and antibiotic prescription, according to risk factors, influenza vaccination status and time after the onset of symptoms (fever, myalgia, headache, shivers, asthenia, cough, nasal discharge...). Data centralization is still in process but final analysis will be available in September.

**Results:** This interim analysis reports 50% of data collected up-to-date. In a 17-week period [week 47 - week 11], during influenza activity, 2 emergency care units included 541 children with clinical symptoms suggesting influenza. The vast majority of IRT were performed between week 1 and week 4 (46.6%). The mean age of the children was  $3.8 \pm 3.1$  years. Children aged between 1 and 5 years accounted for 71.7% of cases. For 68.3% of patients, the onset of symptoms was less than 48h (31.8% < 24h, 17.7% <

12h). High risk children according to French recommendations for influenza vaccine were 8.2%. Among those, only 11.4% were vaccinated. Inaugural acute otitis media and/or pneumonia were diagnosed for 6.3% of children (n=33).

IRTs were positive in 45.3%, negative in 54.2% and not assessable in 0.6% of cases. Diagnosis of influenza was confirmed by IRT in 43.4% children under 5 years old and 54.4% in children more than 6 years old ( $p < 0.04$ ).

In the overall population and for at risk children, according to IRT results, antiviral and antibiotic use is more targeted, i.e. practically no antiviral use and significantly more antibiotic use when IRT negative compared to positive IRT.

	Antiviral prescription	Antibiotic prescription	Parental absenteeism related to child care
Overall population			
Positive IRT	30.5% (73/239)	7.2% (17/235)	17% (38/224)
Negative IRT	0.3% (1/283)	18.2% (50/274)	5.5% (15/273)
	$p < 0.0001$	$p < 0.0002$	$p < 0.0001$
At risk children			
Positive IRT	13.3% (2/15)	20% (3/15)	13.3% (2/15)
Negative IRT	0	66.7% (18/27)	3.7% (1/27)
		$p = 0.003$	$p = 0.3$
Children with acute otitis media and/or pneumonia			
Positive IRT	30.8% (4/13)	76.9% (10/13)	7.7% (1/13)
Negative IRT	0	72.2% (13/18)	17.6% (3/17)
		$p = 0.09$	$p = 0.7$

**Conclusion:** The interim results of this study confirm that IRT can improve the diagnosis of influenza in children, even among children older than 6 years and even at the peak of influenza activity. More targeted influenza management with antivirals and antibiotics occurs when IRT results are available in the overall child population and high risk children. This study also shows that influenza vaccination among high risk children still needs to be improved.

2-014

### Usability of near patient tests for rapid detection of influenza virus of subtype A(H5N1)

Havlickova, M.<sup>1</sup>; Schweiger, B.<sup>2</sup>; Machova, J.<sup>3</sup>; Jirincova, H.<sup>1</sup>; Kyncl, J.<sup>1</sup>

<sup>1</sup>Centre for Epidemiology and Microbiology, National Institute of Public Health, Czech Republic; <sup>2</sup>Robert Koch Institute, Germany; <sup>3</sup>State Veterinary Institute, Prague, Czech Republic

**Introduction:** In view of the risk of the import of human infection with influenza A(H5N1) virus from the initial foci and repeated human exposure from epizootics in Europe, rapid detection of influenza virus H5N1 is highly relevant to both the therapy and anti-epidemic measures. The near patient tests (NPT) or point-of-care (POC) tests have been used with increasing frequency: the time required to perform the assay is usually less than 30 minutes, however the sensitivity of these tests is clearly not satisfactory.

**Methods:** In an experimental model, the kits RapidSignal™ Influenza A/H5 (Organics, Israel), Anigen Rapid H5 AIV influenza Ag Test (Animal Genetics, Korea) and Axiom Avian Influenza Card (GmbH, Germany) were tested. All are immunochromatographic tests for the qualitative detection of haemagglutinin of subtype A(H5). In parallel, the kits QuickVue Influenza A+B test (Quidel, USA) and Now Flu A test (Binax, USA) were performed. They allow for the universal detection of influenza A viruses by using the nucleoprotein as a target. The sensitivity was tested on a set of subtype A/H5 virus strains at dilutions of 0.25 - 25 haemagglutination units (HU) while specificity was tested on strains with heterologous haemagglutinin subtypes (H1, H2, H3, H4, H6, H7, H8, H9, H10 and H11) at a dilution of 1 HU. Rapid tests were carried out according to the manufacturer's instructions. The haemagglutination titer was determined using a 0.5 % suspension of turkey erythrocytes.

**Results:** Two H5 kits showed 100% specificity with none of the heterologous viruses testing positive (0/10). A cross reaction with H1N1 viruses was detected for one of the H5 kits but no reaction with other subtypes was provable. The sensitivity ranged between 1 HU and 25 HU, heavily depending on the virus strain analysed. Thus sensitivity of the kits was rather low from the clinical perspective taking into account the concentration of viruses in respiratory specimens. Generally, higher detection rates were achieved for older variants while the weakest reaction was observed with the strain A/Dk/Vietnam/05. Sensitivity did not exceed 1 HU which is a limitation to the use of the tested kits under field conditions and for direct testing of freshly collected specimens without virus enrichment. Both kits for the detection of type A influenza virus, i.e. QuickVue Influenza A+B test and Now Flu A test, were able to detect all of the tested subtypes with considerably higher sensitivity (at least 0.25 HU).

**Conclusion:** At present, the kits for rapid detection of subtype A(H5) of influenza virus are characterised by high specificity but

low sensitivity. Kits for the detection of type A influenza viruses seem to be more suitable for rapid detection of influenza virus from suspected cases. The "influenza A yes/no" response is of high diagnostic relevance when a human case of avian influenza is suspected. Further development of rapid H5 tests with higher sensitivity will be greatly relevant to routine practice.

2-015

### Differential presentation of clinical symptoms in relation to influenza virus type and subtype

Fleming, D.M.; Elliot, A.J.

Royal College of General Practitioners, UK

**Background:** Influenza can manifest itself as many different clinical symptoms and diagnoses. We have previously shown that the clinical diagnoses of influenza-like illness, common cold, otitis media and acute bronchitis are all diagnosed during the period of circulation of influenza viruses in the community.

**Aims:** We aimed to determine whether different influenza types i.e. A and B, or different influenza subtypes i.e. H1N1 and H3N2, were associated with different clinical presentations to community-based GPs.

**Methods:** We utilised clinical incidence data derived from the Royal College of General Practitioners Weekly Returns Service sentinel general practitioner network for a range of respiratory diagnoses including those mentioned above. In order to determine circulating virus periods, we used laboratory reports for influenza accessed from the Health Protection Agency; these reports were differentiated into type and subtype.

**Results/Discussion:** Results from our analyses will be presented and discussed with emphasis on improving the recognition and diagnosis of influenza infections in the community in the absence of confirmatory laboratory testing.

### Support strategies for molecular clinical diagnostic testing for influenza among public health laboratories in the United States

**Lindstrom, Stephen;** Johnson, J.R.; Villanueva, J.; Shu, B.; Emery, S.; Wu, K.H.; Berman, L.; Winter, J.; Klimov, A.

*Centers for Disease Control and Prevention, USA*

**Background:** Procedures for detection and characterization of influenza viruses using real-time RT-PCR (rRT-PCR) were developed at the Centers for Disease Control and Prevention (CDC), U.S.A. and implemented for rapid testing to identify individuals infected with seasonal human influenza as well as test specimens from suspect H5N1 human cases. Assays were designed for universal detection of all influenza type A and type B viruses as well as identification of the HA genes of influenza A viruses of human health significance including contemporary human H1 and H3, as well as Eurasian-lineage avian H5 viruses. In order to provide support to public health laboratories (PHLs) within the USA, detailed protocols including primer and probe sequence information have been made available to public health laboratories (PHLs) since 2004. However, variability in assay performance at each laboratory is observed due to sourcing of oligonucleotide primers and probes from a number of different manufacturers, as well as independent optimization and validation of the assay in the laboratories. To reduce performance variability and improve support for influenza surveillance, efforts were initiated for CDC to provide standardized reagents and optimized procedures to PHLs. In order to improve pandemic preparedness and enhance domestic testing capacity to rapidly detect individuals that may be infected with H5N1 HPAI, the Influenza Division applied to the United States Food and Drug Administration (FDA) for approval to distribute primer/probe sets specific for Asian H5 HPAI for clinical testing within the United States. Following approval of the Influenza A/H5 (Asian lineage) Virus Real-time RT-PCR Primer and Probe Set by the FDA, these reagents were made available to over 100 laboratories. For the purpose of expanding support to PHLs by providing qualified reagents for differential diagnosis of human seasonal influenza, a clinical study was undertaken to compare the performance of the CDC Human Influenza Virus Real-time RT-PCR Detection and Characterization Panel (rRT-PCR Flu Panel) with virus culture of human respiratory specimens from patients presenting with influenza-like illness.

**Study Design:** The clinical performance evaluation study was designed to test human respiratory specimens from patients who presented with influenza-like illness (ILI) at four (4) U.S. PHL clinical testing locations. Collected specimens were tested by prospective routine "Gold Standard" virus culture testing along with the rRT-PCR Flu Panel procedure.

A total of 515 specimens were collected and tested during the 2006/2007 influenza season for this clinical study which

included throat swabs (23), nasal swabs (9), combined nasal and throat swabs (10), naso-pharyngeal swabs (417), throat and naso-pharyngeal swabs combined (15), nasal aspirate (13), and broncho-aveolar lavage (2). There were twenty-four (24) culture isolates tested, and two (2) specimens were reported as unknown specimen types or not reported. Data were collected and stratified to ensure all age groups were included in the testing. The numbers of representative specimens from previously described sources were collected from the following age groups: 0-16yrs (108), 17-54yrs (261), greater than or equal to 55yrs (91), and not reported (55). In general, the majority of specimens were nasopharyngeal swabs from patients aged 17-54 years.

**Results & Discussion:** Four hundred and eighty-two rRT-PCR results were confirmed by "Gold Standard" testing. Four hundred and thirty-two specimen results were confirmed by virus culture, and 50 specimen results required confirmatory bi-directional sequencing at the CDC Influenza Division laboratory. Assay performance for each target included in the rRT-PCR Flu Panel (InfA, InfB, AH1, AH3) was measured and defined as values of clinical sensitivity, clinical specificity, positive predictive value (PPV), negative predictive values (NPV), and percent agreement values. The clinical sensitivity values for all markers were greater than 95% and the clinical specificity values for all markers were greater than 97%. PPVs and NPVs for human influenza targets were greater than 96% while percent agreement values were all greater than 97%. The clinical performance of the CDC rRT-PCR Flu Panel in a multi-site evaluation was very high, demonstrating this device to be a very specific and sensitive clinical diagnostic tool for detection and differentiation of seasonal human influenza viruses in human specimens.

2-018

### Detection of human (A/H1, A/H3 and B) and avian (A/H5) influenza virus by a multiplexed bead suspension array system

**Winter, J.;** Shu, B.; Berman, L.; Emery, S.; Klimov, A.; Lindstrom, S.

*Influenza Division, Centers for Disease Control and Prevention, USA*

**Background:** Molecular diagnostic methods based on real-time reverse transcriptase polymerase chain reaction PCR (rRT-PCR) for the detection and characterization of human and animal influenza viruses have become standard procedures in many laboratories. However, genetic variability due to virus evolution can limit the sensitivity of detection and may require periodic modifications of rRT-PCR primers and probes. Also, the implementation of this method may be limited due to logistical reasons such as equipment cost or reagent availability. In this

study, we addressed these limitations by investigating the use of established diagnostic rRT-PCR primers and probes designed for the detection and characterization of human (A/H1, A/H3 or B) or avian (A/H5) influenza viruses by a multiplexed endpoint detection assay based on the Bio-Plex system. We addressed the issue of genetic variability by investigating the use of multiple, sequential probes binding to the same amplicon for confirmation or discrimination of human or avian influenza viruses. We also looked at the multiplexed bead suspension array system as a complementing and/or alternative method for analyzing rRT-PCR products, since many diagnostic and surveillance laboratories have already implemented this platform for routine protein-based assays.

**Objective:** The purpose of this study was to adapt established rRT-PCR primers and probes to the Bio-Plex bead system and maintain/improve the assay's sensitivity by combining multiple probes with overlapping and non-overlapping sequences in a single bead-based assay.

**Methods:** Multiple oligonucleotide primer sets designed for universal detection of type A and type B influenza as well as characterization of seasonal human A/H1 and A/H3 or avian A/H5 influenza viruses by rRT-PCR were used to amplify specific gene targets from total RNA extracted from grown viral material. Amplified RT-PCR products were subsequently analyzed by hybridization to target-specific oligonucleotide probes coupled to fluorescence-labelled polystyrene beads (Bio-Plex Suspension Array System). Positive control materials were designed by synthesizing oligonucleotides that were complementary to the detection probe sequences. Influenza virus from specimens or controls was detected by the rRT-PCR protocol which has been established at the Centers for Disease Control and Prevention.

**Results:** The Bio-Plex suspension array system was highly specific for the detection and discrimination of human influenza virus type A (subtype H1 and H3), influenza virus type B or avian influenza virus (A/H5). Biotinylation of oligonucleotide primers did not interfere with the assay's performance. Hybridization assays were optimized to demonstrate limits of detection between 10 and 100 fmol control-oligonucleotides. Multiplexed hybridization analysis of RT-PCR amplified products did not affect the detection limits of the assay. In addition, multiplexing of hybridization probes binding within the same amplicon did not affect the detection limits of the assay.

**Conclusion:** The bead suspension array system for detection and characterization of human and avian influenza demonstrated reaction sensitivity and specificity comparable to previously established rRT-PCR methods. Oligonucleotide primers and probes specifically designed for rRT-PCR can be easily adjusted for use in a multiplexed bead-based assay for the typing and subtyping of influenza virus.

2-019

## Improved diagnosis of influenza virus infection using a multiplex PCR

**Ortiz de Lejarazu, R.<sup>1</sup>; Tenorio, Alberto<sup>1</sup>; Eiros, J.M.<sup>1</sup>; Bermejo-Martin, J.F.<sup>2</sup>; Castrodeza, J.<sup>3</sup>; Vega, T.<sup>3</sup>**

<sup>1</sup>Hospital Clinico Universitario Valladolid, Spain; <sup>2</sup>Hospital Clinico Universitario Valladolid, Spain; <sup>3</sup>Consejeria de Sanidad Castilla y León, Spain

**Introduction:** Acute respiratory infection is the first cause of infectious pathology in humans. Isolation, detection and subtyping of influenza viruses are relevant tools for knowing the epidemical evolution, being in addition necessary for designing the influenza vaccine each season. However, today there is no consensus definition for influenza cases from the ESWi or any other international body. This makes early virologic diagnostic more valuable when alerting the sentinel network of influenza surveillance on the appearance of new cases.

**Objectives:** 1) To evaluate the application of an RT-nested PCR in the diagnosis and subtyping of influenza viruses. 2) To employ this method to study the epidemic evolution of influenza viruses during the 2007-2008 season in the region of Castilla y León, Spain.

**Methods:** Nasopharyngeal swabs collected by the physicians from the sentinel network of Castilla León were analysed for the presence of influenza virus. The samples were processed in parallel by the shell vial classic culture method and by PCR. Culture was done using MDCK cells; revealing was performed by using anti-influenza A and B monoclonal antibodies, with further reading under fluorescence microscope. For molecular diagnosis, an initial RT-PCR step was employed, followed by a nested PCR designed for the multiple detection of influenza A, influenza B, influenza C, respiratory syncytial virus (RSV) type A and type B and adenovirus. The samples that were positive for influenza A by PCR were subtyped by using the same method. A microarray-based assay for the simultaneous diagnosis of 14 respiratory viruses (Clinical Array, Genomica S.A.U®, Madrid, Spain) was tested on 12 samples positive for influenza A by PCR, in order to study the correlate between both methods.

**Results:** 84 samples of the 2007-2008 seasons were analyzed. 6 influenza virus type A along with 15 influenza virus type B were isolated by using the Shell-vial method. On the contrary, the employed PCR based method was able to detect 28 cases of influenza virus A (28 H1), 23 influenza virus B, 1 influenza virus C, 4 adenovirus and 1 RSV type B. When the positive results by culture were compared with the results by PCR, the correspondence was 100%. Our PCR was able to detect 5 co-infections: 2 influenza virus A + adenovirus, 1 influenza virus B + adenovirus, 1 influenza virus A + influenza virus B and 1 influenza virus B + influenza virus C. The twelve samples analysed in parallel by PCR and microarray showed a correspondence of 100%. The microarray method was able to detect in addition co-infection with RSV A in 4 samples

and co-infection with coronavirus 229E in one sample.

**Conclusions:** In our work, the employed PCR showed excellent correspondence with the positive results by culture. The PCR method showed an increased ability to detect influenza virus, since the number of influenza cases detected increased twofold with this approach. The use of PCR-based methods in combination with culture methods, which allows recovering viral specimens, has allowed for the increase in the number of positive diagnostic results. This is important mainly in the early stages of the influenza season in those cases when the virus does not grow in the cultures, due to a poor viral load or to a deficient shipment. Moreover, the employment of a PCR multiplex method can aid the differential diagnosis with other respiratory viruses which can produce influenza-like symptoms. In addition, the PCR method allowed us to obtain the viral subtype, and was able to detect co-infection with other circulating respiratory viruses. In conclusion the use of a molecular method combined with the classical culture approach provides meaningful advantages from the point of view of the virological diagnosis and the epidemiological surveillance. The molecular methods lead to faster performance, provide higher sensibility and target a wider diagnostic spectrum, complementing the traditional culture methods, which are nonetheless still necessary for growing virus for vaccine design purposes.

2-021

### The art of viewing and influenza - its importance for diagnosis

**Vogel, Georg E.<sup>1</sup>; Komm, C.<sup>2</sup>; Kunkel, R.<sup>2</sup>; Lorenz, H.<sup>2</sup>; Manych, M.<sup>2</sup>; Möller, R.<sup>2</sup>; Schöttler, M.<sup>2</sup>; Vasold, M.<sup>2</sup>**

<sup>1</sup>Internal Practice, Germany; <sup>2</sup>

#### "A picture paints a thousand words."

Increasingly, the focus of medical observation is on analytical parameters, and the patient is disappearing from the doctor's sight. For a long time this sight was all that the doctor had, and it was the basis of his/her diagnosis and treatment. In the 19<sup>th</sup> Century, doctors compared the physiognomies of healthy and sick patients; the development of this sight was part of medical training. When great artists depict what they have observed or felt personally, the result is an especially incisive picture. Among others, Edvard Munch, Egon Schiele and Olaf Gulbransson have created works of art that reveal in a highly impressive manner the suffering and illness of human beings. At the Mount Sinai Medical School in New York the "art of viewing" has been taught since 2006. With the help of paintings, medical students are learning again how to recognize the body's diverse signals.

Very early on we were struck by the suffering physiognomy of

influenza patients with their fearful expressions reflecting the premonition of an impending threat. Since January 1999, when we first started treating our patients antivirally, we have been photographing their facial expressions in the sudden onset stage and during the course of treatment. During treatment with neuraminidase inhibitors – sometimes even within a few hours – it was possible to observe how the blocking of virus replication was directly and clearly reflected in the patients' physiognomies. We were able to measure this process analytically in 210 influenza patients through monitoring of the humoral inflammation status. Our experience to date is presented with exemplary photographic documentation and its artistic translation focussing on the essence of the pictures, in the true sense of the "art of viewing". This confirms what the well-known contemporary painter Daniel Richter has observed: "Painting is preserved time". We now know more about these conditions and can explain them as an infection of the host cell in the course of virus replication, the release of cytokines and disturbed microcirculation. The interactions taking place during a simultaneous viral and bacterial attack are also known (Rolf Zinkernagel, 2006).

We have summarised all of this knowledge under the motto, "Only he who knows the past can create the future". We consider it crucial for the quality of patient care that up-to-date knowledge on the virus, host cell, immune system, diagnosis and antiviral treatment be combined with the powers of observation of earlier times. Medicine has to remain a helping discipline – it is more than merely a mathematical and technical experimental science (Frank Nager, 2002).

- Painting is preserved time ([www.daniel-richter.com](http://www.daniel-richter.com))
- Painted Results (since January 1999) of influenza patients with their "sudden onset" and cytokine reaction and after therapy with neuraminidase inhibitors (Dr. Regula Kunkel).

2-022

### The family doctor: key figure and model of the future for patient care regarding influenza and other respiratory tract infections

**Vogel, Georg E.<sup>1</sup>; Back, T.<sup>2</sup>; Blasini, R.<sup>2</sup>; Heckler, R.<sup>2</sup>; Komm, C.<sup>2</sup>; Lange, W.<sup>2</sup>; Manych, M.<sup>2</sup>; Möller, R.<sup>2</sup>; Schäfer, B.<sup>2</sup>; Schöttler, M.<sup>2</sup>**

<sup>1</sup>Internal Practice, Technical University, Germany; <sup>2</sup>

"Once there was the family doctor who knew everything about us and who knew and treated all the illnesses of every member of the family..." (Weltwoche Nr. 14.07, S.67).

The importance of the family doctor where influenza is concerned applies to the whole chain of action, from prophylaxis to treatment. The introduction of the computer with its connection

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to the Internet into the family doctor's practice makes it possible to obtain daily updated surveillance reports. Through timely observations of a beginning epidemic, prophylactic vaccination is still possible and sensible. Peter Palese recommends it for everyone – children, adults and the aged. Surveys among patients show that the family doctor and his/her assistants have the greatest influence on the patients' willingness to be vaccinated. In our own practice we started out in 1993 with a vaccination rate of 15%, and today, through a resolute approach that includes consultation and information, have increased this rate to 85%. We thus obtained a reduction in colds of 39% among the vaccinated patients. (number of vaccinations 2006/2007: 915 total number of vaccinated patients).

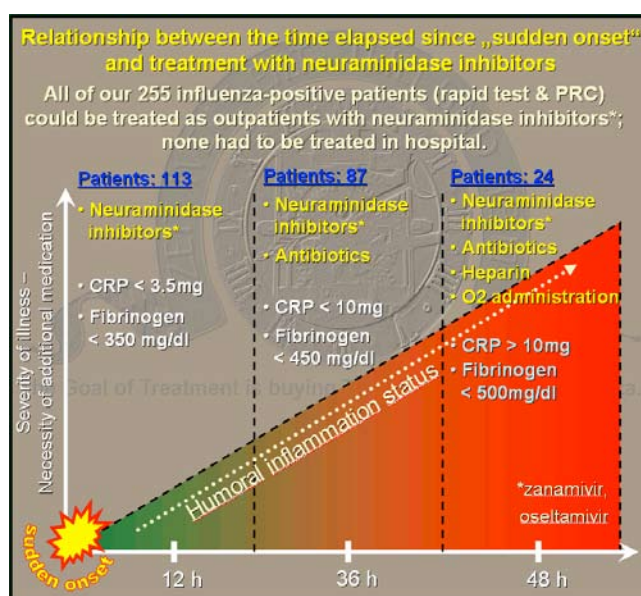
Since January 1999 we have gained experience in the treatment of 225 influenza patients (male and female) between the ages of 1 and 96. (When we treated the first patient on January 29, 1999 we did not yet have a rapid test available.) A reliable diagnosis was obtained with the rapid test QuickVue, Quidel, which was confirmed by an RT-PCR (State Health Department of Lower Saxony, Hanover, Germany, Dr. R. Heckler). The course of the illness following the typical "sudden onset" was monitored with the so-called humoral inflammation status (sum of the C-reactive protein and fibrinogen levels). The result was: "The goal of treatment is buying time". If it is possible to diagnose the patient's illness on the first day of infection and treat him/her immediately (!) with neuraminidase inhibitors, the immune system can stop the viral infection.

Rolf Zinkernagel has described the interaction between viral and bacterial infections. These data have convinced us that every case of community acquired pneumonia is preceded by damage to the epithelium of the airways caused by a virus (often by influenza virus). Through our timely diagnosis and the described monitoring of the course of the viral infection we gain time for an early bacterial swab and antibiogram. In this way we are able to recognize in time the threatening resistances of *Haemophilus parainfluenzae* and *Staphylococcus aureus* (at present approximately 40%) and choose a suitable antibiotic, should this be necessary following an increase in the CRP level to above 3.5 mg/dl. We have reduced the use of antibiotics to less than 50%. Considering the burden on health systems – not only in Germany – through antibiotic resistance, these are important results.

Nine out of 10 upper respiratory tract infections are caused by viruses; 10-15% of these are due to influenza. We have followed steadfastly a statement made in the 1950s and have extended our approach to include other "common cold" viruses: "If you understand influenza, you understand the whole of virology". Therefore, in addition to the influenza rapid test we are now also using Quidel's RSV rapid test and have had positive results of our patients (16% influenza and 6.5% RSV and other viruses). In 1408 patients we realized that the RSV infection was anything but trivial and self-limiting. In future it will be possible to prove the presence of a dozen other cold viruses with a rapid test ([www.fda.gov/bbs/topics/NEWS/2008/NEW01770.html](http://www.fda.gov/bbs/topics/NEWS/2008/NEW01770.html)). In 37%

of these cases the humoral inflammation status was exceeded, with an increase in the CRP level to above 3.5 mg/dl, and an antibiotic was necessary. Higher treatment costs are more than outweighed by the successful treatment of a community acquired pneumonia recognized early enough.

The role of the family doctor has been mentioned explicitly in the 2006 Pandemic Plan for Germany. This finds its expression in Neil Ferguson's suggestion, "Act locally, stop globally". We are convinced this will be possible if the practice next door to ours acts exactly as we do.



2-023

### Usability of SRH assay to titration H5 antigen

**Picciarella, S;** Gentile, C.; Mennitto, E.; Manini, I.; Alberini, I.; Montomoli, E.

University of Siena, Italy, Department of Physiopathology, Experimental Medicine and Public Health, Italy

Single Radial Haemolysis (SRH) was developed in 1975 and is routinely used to detect influenza-specific and rubella IgG antibodies. It has a good correlation with Virus Neutralization (VN) and is EMEA approved. SRH testing has recently been used to detect human antibodies to avian influenza viruses. To validate the usefulness of SRH to detect antibody levels against pandemic H5 strains, we used human sera from a pandemic study and carried out SRH applying ICH Harmonised Tripartite Guideline criteria: specificity, accuracy, repeatability-precision, intermediate-precision, linearity and robustness. Thirty sera were

used from adults vaccinated in a Phase II clinical trial for A/H5N1/Vietnam/1194/04 vaccine, only analysed by VN and HI, including ten with negative Ab-titre, ten with a high positive Ab-titre and ten with a low positive Ab-titre (43 days post vaccination). ICH Harmonised Tripartite Guideline criteria were used for analysing: specificity: titration of 5 duplicates of all sera and controls; accuracy: percentage of difference between the geometrical average of the haemolysis area of the undiluted positive control sera and the area of haemolysis of the same sera diluted at 1:2 in Phosphate Buffered Saline; repeatability-precision: 6 duplicates with the same samples; intermediate-precision: testing the same sera three times by two people; linearity: evaluating pool sera with a high positive Ab-titre diluted in negative serum (1:1, 1:2, 1:4, 1:8 and 1:16); robustness: studying the impact of two independent parameters (virus concentration, incubation period of sera). All parameters were within our established limits and according to ICH-HTG criteria. Specificity criteria were 100%, linearity criteria had a correlation coefficient of  $> 0.96$ , repeatability-precision and intermediate-precision coefficients of variation were both  $< 25\%$ , and the antibody response for robustness was similar in every condition tested and the difference was never over 15%. Thus, SRH is effective for evaluating the immunogenicity of an H5N1 strain vaccine.

2-024

#### Development and characterisation of monoclonal antibodies to H5N1 influenza virus

Linke, S.<sup>1</sup>; Neubauer, K.<sup>1</sup>; Dorner, B.<sup>2</sup>; Dorner, M.<sup>2</sup>; Pauli, G.<sup>2</sup>; **Schweiger, B.**<sup>1</sup>

<sup>1</sup>Robert Koch-Institut, National Reference Centre for Influenza, Berlin, Germany; <sup>2</sup>Robert Koch-Institut, Centre for Biological Safety, Berlin, Germany

The outbreaks of highly pathogenic avian influenza virus (AIV) of the H5N1 subtype in patients mainly in Asia have put increased focus on the role of rapid antigen detection systems. There is a growing demand for H5-specific tests, which do not need laboratory operation. Reports suggest that existing rapid antigen detection tests for AIVH5 in patients have a poor sensitivity. As we could show in a study undertaken with the Czech National Influenza Centre, the sensitivity of H5 rapid tests analysed so far is lower than for commercially available human influenza A test systems. Some products miss data regarding specificity, while others show cross-reactivity with influenza subtype H1. Furthermore, information on their application for H5-infected patients is limited. In order to improve the tests we generated and characterised monoclonal antibodies (mAbs) to influenza virus subtype H5. Mice were immunized with inactivated AIVH5N1 A/

Whooper swan/Germany/R65-2/06. After fusion of the murine splenocytes with a myeloma cell line 5 mAbs were selected, which exclusively detect AIVH5 viruses in a direct ELISA-assay. These antibodies were further characterised by other methods like Sandwich-ELISA, immunofluorescence assay (IFA), virus neutralisation assay (NT) and Western blot. Three mAbs detect AIV H5N1 infected cells by IFA, but all generated antibodies lack neutralisation properties. The first results by Western blot analysis show that most mAbs detect epitopes of the HA protein. Sandwich-ELISA studies reveal that all attained antibodies detect the antigen in a virus dilution and are therefore appropriate for testing development. At present, the specificity of the mAbs is assessed for different AIVH5 viruses and AIV belonging to other HA subtypes as well as relevant viruses and bacteria which can induce respiratory infections in patients. In conclusion, the inactivated influenza virus subtype H5N1 represents an efficient immunogenic stimulant for generating specific mAbs to AIVH5. Testing their ability to accurately diagnose H5-infections in a rapid antigen detection assay is in progress. Linke, S. and Neubauer, K. have contributed equally to the manuscript.

2-025

#### Evaluation of avian influenza diagnostics

**Myers, Christopher**; Faix, D.J.; Russell, K.L.

Naval Health Research Center, USA

The Department of Respiratory Disease Research at the Naval Health Research Center (NHRC), San Diego, California, conducts population-based surveillance for febrile respiratory illness (FRI) at eight US military basic training centers. NHRC also supports surveillance on large-platform US Navy ships and several clinics along the US-Mexico border. More recently, NHRC began surveillance at three local clinics that serve the dependents of active-duty military personnel. These diverse samples are shipped to NHRC and tested by molecular- and culture-based assays that can identify nearly 40 different respiratory samples. In more than 10 years of surveillance, NHRC has managed to collect over 500,000 samples. The United States National Strategy for Pandemic Planning, issued by the White House on November 1, 2005, assigned the Department of Defense (DoD) the task of evaluating promising technologies with the ability to detect and differentiate highly pathogenic avian influenzas from seasonal strains. With the support of the DoD-Global Emerging Infections Surveillance and Response System (DoD-GEIS), NHRC collected information on available diagnostic tests for avian influenza and selected more promising diagnostics for further study based on strengths in one or more of these areas: methodology, deployability (shipboard and field), ease of use,

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and cost. The ability to distinguish avian influenza from seasonal strains was considered crucial. Evaluation of diagnostics was done in three stages: (1) evaluation against grown control samples; (2) evaluation against clinical samples; and (3) field and/or point-of-care evaluation. For the first two stages, NHRC can leverage its archive of respiratory samples and its ongoing surveillance for recent influenza samples. For the last stage, NHRC can use the dependent clinics as testing sites and/or collaborate with overseas partners such as Naval Medical Research Units 2 and 3 located in Jakarta, Indonesia, and Cairo, Egypt, respectively. Evaluation of several diagnostics is currently under way, with different diagnostics at different stages. The methodologies and strengths of differing technologies will be discussed.

2-026

### Quality assessment of molecular diagnostic methods for influenza detection and characterization among state public health laboratories in the United States

Wu, Kai-Hui<sup>1</sup>; Johnson, J.<sup>2</sup>; Grosso, L.<sup>3</sup>; Humes, R.<sup>3</sup>; Shu, B.<sup>2</sup>; Winter, J.<sup>2</sup>; Berman, L.<sup>2</sup>; Lindstrom, S.<sup>2</sup>; Klimov, A.<sup>2</sup>

<sup>1</sup>Influenza Division, Centers for Disease Control and Prevention, USA; <sup>2</sup>Influenza Division, CDC, USA; <sup>3</sup>Association of Public Health Laboratories, USA

**Background:** Molecular diagnostic methods have recently become the method of choice for many public health laboratories in the U.S. for detection of influenza and other infectious diseases. Because the state public health laboratories (SPHLs) are the first line for the detection and diagnosis of infections of seasonal and novel influenza strains in the U.S., their performance is critical for implementation of an appropriate public health response. However, the ability of domestic SPHLs to accurately detect and characterize influenza virus using molecular methods has not been evaluated. In order to assess the U.S. domestic capacity to accurately identify influenza in human cases at the state level, a quality assessment (QA) exercise was initiated by the Influenza Division of the U.S. Centers for Disease Control and Prevention (CDC) and organized with the cooperation of the Association of Public Health Laboratories (APHL). The goal of the QA was to evaluate the capability of 50 SPHLs in the U.S. to detect and characterize seasonal human influenza viruses and avian H5 influenza viruses using molecular diagnostic methods.

**Methods:** The QA was a voluntary exercise available to all 50 U.S. SPHLs. Participating SPHLs were provided with panels of 9 blinded simulated respiratory specimens with clinical case history to evaluate using their molecular diagnostic testing algorithms. Blinded simulated specimens contained cultured human epithelial cells (A549) with or without inactivated influenza viruses. Positive simulated specimens contained varying concentrations

of influenza human A(H1), A(H3), avian A(H5), or influenza B viruses. Prior to distribution, samples were quality control tested for purity and for influenza virus identification. Laboratories were instructed to process all samples as clinical diagnostic specimens using BSL-2 laboratory precautions and perform all manipulations of samples within Class II (or higher) biological safety cabinets. All samples were to be processed as a single batch of "specimens" and tested within seven business days of receipt using the validated molecular diagnostic methods currently used in their laboratory. Test results and conclusions for each sample were recorded on a provided worksheet and submitted electronically to the Influenza Division at CDC. Additionally, all information regarding test parameters including nucleic acid extraction methods, equipment and commercial enzyme kits were also collected.

**Conclusion:** To date, 38 of 50 laboratories have enrolled in the study. Aggregate laboratory results as well as compiled survey information will be presented after completion of the study in May 2008. Results of the QA exercise will be used to estimate the capability of U.S. SPHLs to accurately detect and identify human seasonal as well as Eurasia-lineage avian HPAI. Results will be useful for CDC and APHL in addressing the needs of SPHLs and prioritizing resources for support.

2-027



### Predictive accuracy of different clinical definitions of influenza in the community setting

Kovar, Jana<sup>1</sup>; Cowling, B.J.<sup>2</sup>; Fang, V.J.<sup>2</sup>; Chang, K.H.<sup>2</sup>; Peiris, J.S.M.<sup>2</sup>; Bermingham, A.<sup>3</sup>; Edmunds, J.<sup>3</sup>; Johnson, A.<sup>1</sup>; McMichael, A.<sup>4</sup>; Nazareth, I.<sup>5</sup>; Nguyen-Van-Tam, J.S.<sup>6</sup>; Watson, J.M.<sup>3</sup>; Zambon, M.<sup>3</sup>; Hayward, A.C.<sup>1</sup>; Leung, G.M.<sup>2</sup>

<sup>1</sup>University College London, UK; <sup>2</sup>The University of Hong Kong, Hong Kong; <sup>3</sup>Health Protection Agency, UK; <sup>4</sup>MRC Human Immunology Unit, UK; <sup>5</sup>MRC General Practice Research Framework, UK; <sup>6</sup>University of Nottingham, UK

**Background:** Reliable clinical diagnosis of influenza is essential for appropriate patient triage and management, and the control and mitigation of community spread in the case of a pandemic. Accurate diagnosis is often difficult given the non-specificity of influenza symptoms. Previous studies indicate that the presentation of influenza is age specific. In addition, there is scarce observational data based on natural infections in the community setting, with the most important knowledge gap among children under the age of 12 years. We assessed the predictive performance of different clinical definitions first using data from a large community study in Hong Kong to derive a prediction algorithm, and then validating it using data from the

Flu Watch community study in England.

**Method:** Symptoms of patients presenting influenza-like illness (ILI) (n=998) to 30 outpatient clinics in Hong Kong were recorded. Influenza infection was confirmed using viral culture or RT-PCR on nasal and throat swabs. We used multivariable logistic regression to derive a prediction model of influenza infection. We validated the model using data from the Flu Watch study in England, in which 607 initially well participants in 241 households were prospectively followed over the winter 2006/07. Influenza was confirmed by RT-PCR on nasal swabs during reported episodes of ILI. In both Hong Kong and England, the predominant circulating strain of influenza during data collection was H3N2.

**Results:** Of the 998 patients in the derivation cohort, 32% met the "fever plus cough/sore throat" definition of ILI and 19% (n=186) had laboratory confirmed influenza (RT-PCR of nasal & throat swabs). Preliminary findings indicate that fever, cough and coryza were the best independent predictors of influenza infection in both adults and children. A model based on these three symptoms/signs in combination had an overall sensitivity of 60%, specificity of 79% and an area under ROC curve of 0.77. The model maintained predictive performance on the Flu Watch data and detailed validation will be presented. We will also present prediction models for children and adults separately.

**Conclusions:** Our findings suggest that in addition to fever and cough, coryza was similarly predictive of influenza infection. Whilst the US CDC advocates the use of fever and cough or sore throat as the definition of ILI, our findings suggest that sore throat is not independently predictive of influenza infection. The development and validation of prediction models can broaden the evidence base for accurate definitions of ILI for use in both research and clinical settings.

2-028

### Clinical and laboratory findings of influenza A (H5N1) human cases in the Tangerang district, Indonesia

**Lokida, Dewi<sup>1</sup>; Tintin, S.<sup>2</sup>; Udjiani, <sup>2</sup>; Ariani, <sup>2</sup>; Mamahit, M.J.N.<sup>2</sup>**

<sup>1</sup>Tangerang Hospital, Indonesia; <sup>2</sup>Tangerang District Hospital, Tangerang, Indonesia

By the end of March 2008, 132 H5N1 confirmed cases had been reported in Indonesia, of which 21 (12%) were from Tangerang district, a suburb with 4.5 million inhabitants in west and south-west Jakarta. The case fatality proportion (90%) was significantly higher than the national case fatality proportion. The mean age of the cases was 22.5, ranging from 3 to 50 years old. Male to female ratio was 1: 1.6. Among the cases were 2 family clusters, each consisted of 2 family members, but no human-to-human transmission was clearly detected (NIHRD). The first H5 cases

and cluster in Tangerang (also in Indonesia) occurred in June 2005 involving a father and his daughter. Risk-associated contacts were only reported in 13 (61.9%) of the cases, consisting of 38.4% direct contact with dead/sick chicken, 7.6% contact with poultry products, 7.6% lived or worked close to poultry market and 46.1% had a backyard chicken (33.3%). All of the patients had sought medical assistance as early as day 1 (mean: 4 days, CI 95%: 2.79-5.20) of their illness before they were suspected of H5 infections at one of Tangerang's avian influenza network hospitals (mean: 6.6 days, CI 95%: 5.44-7.78). Initial diagnoses varied from seasonal influenza, atypical pneumonia, bacterial pharyngitis, diarrhea, to dengue and typhoid fever. Clinical manifestations included high fever (100%), cough (100%), dyspnea (100%), dizziness (92%), headaches (86%), myalgia (66%), diarrhea (64%), athralgia (60%) coryza (58.8%) and also sore throat (53%). In the chest X-ray, all the patients had pneumonia with or without pleural effusion or pulmonary oedema. Complete blood count results revealed leukopenia (88%), lymphopenia (92.8%) and thrombocytopenia (94.5%): leukocyte count (mean: 3061/iL, CI 95%: 2378.5-3817.6), lymphocyte absolute count (mean: 736/iL, CI 95%: 453.0-1021.4) and thrombocyte count (mean: 121,611/iL, CI 95%: 110,171.4-133,050.8). At terminal stage of illness, most patients died from respiratory failure and multiple organ failure. There were significant differences in clinical manifestation and hematology results between avian influenza, human influenza, dengue fever and typhoid. Between avian influenza and human influenza: dyspnoea (P < 0.01), chest pain (P < 0.01) leukopenia (P: 0.058), lymphopenia (P < 0.001), thrombocytopenia (P < 0.001). Between avian influenza and dengue fever: dizziness (P: 0.0011), myalgia (P: 0.031), diarrhea (P: 0.008), leukopenia (P: 0.0024), lymphopenia (P: 0.0063). Between avian influenza and typhoid fever: myalgia (P: 0.0053), athralgia (P: 0.0016), cough (P < 0.001), coryza (P < 0.001), sore throat (P < 0.001), leukopenia (P < 0.001), lymphopenia (P < 0.001), and thrombocytopenia (P < 0.001). A high incidence rate of H5 influenza in Tangerang indicates a sustained H5 transmission in poultry as well as a high awareness of this disease among clinicians in the AI network hospitals. However, cases might still be under-reported as many areas are geographically remote from health services, a substantial number of people have financial problems in seeking medical care, and awareness among private practice clinicians is still inadequate. High CF proportion was due to late diagnosis and consequently ineffective antiviral treatment. Training has been provided for health care providers to be aware of early presentations of H5 infections, including clinical laboratory profiles and risk-associated poultry contacts in order to distinguish between H5N1 infection and its differential diagnoses during early illness when antiviral (in the Indonesia case, oseltamivir) is expected to be effective.

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# **A randomized trial of face masks and hand hygiene to prevent influenza transmission in households**

**Cowling, B.J.<sup>1</sup>; Fung, R.O.P.<sup>1</sup>; Fang, V.J.<sup>1</sup>; Cheng, K.Y.<sup>1</sup>; Lam, C.L.K.<sup>1</sup>; Chan, K.H.<sup>2</sup>; Seto, W.H.<sup>3</sup>; Yung, R.<sup>4</sup>; Chu, D.W.S.<sup>3</sup>; Chiu, B.<sup>5</sup>; Lee, P.<sup>6</sup>; Chiu, M.C.<sup>7</sup>; Lee, H.C.<sup>8</sup>; Uyeki, T.M.<sup>9</sup>; Houck, P.M.<sup>10</sup>; Peiris, J.S.M.<sup>2</sup>; Leung, G.M.<sup>1</sup>**

<sup>1</sup>Department of Community Medicine and School of Public Health, the University of Hong Kong, China; <sup>2</sup>Department of Microbiology, the University of Hong Kong, China; <sup>3</sup>Hospital Authority, Hong Kong, China; <sup>4</sup>Centre for Health Protection, Hong Kong, China; <sup>5</sup>Hong Kong Sanatorium and Hospital, Hong Kong, China; <sup>6</sup>St Paul's Hospital, Hong Kong, China; <sup>7</sup>St Teresa's Hospital, Hong Kong, China; <sup>8</sup>Hong Kong Baptist Hospital, Hong Kong, China; <sup>9</sup>Influenza Division, CDC, Atlanta, Georgia, USA; <sup>10</sup>Division of Global Migration and Quarantine, National Center for Preparedness, Detection and Control of Infectious Diseases, CDC, USA

**Background:** There are sparse data on the efficacy of non-pharmaceutical interventions to prevent influenza spread. Following successful completion of a pilot of 128 households in 2007, [1] we implemented a large study of the efficacy of face masks and hand hygiene to reduce within-household transmission (January to September 2008).

**Methods:** We conducted a cluster randomized controlled trial of households (with at least 2 other members who had remained asymptomatic of influenza-like illness in the previous 2 weeks) where an index subject presented an influenza-like illness of <48-hour duration. After confirmation by a rapid test, the household was randomized to 1) control group or 2) hand hygiene or 3) hand hygiene plus surgical masks. Households were visited within 36 hours, and again 3 and 6 days later. Nose and throat swabs were collected from index subjects and household contacts at each home visit and tested by RT-PCR and viral culture. The primary outcome measure was laboratory confirmed influenza in a household contact; the secondary outcome measure was clinical influenza (by self-reported symptoms) defined as at least 2 of fever of 37.8 °C, cough, headache, sore throat, aches or pains in muscles or joints.

**Interim results and anticipated conclusions:** Interim analysis of the first 104 households recruited by March 31, 2008 indicated that the overall secondary attack ratio of clinical influenza among household contacts was 16%. In multivariable analyses we found that household contacts in the hand hygiene group had an estimated odds ratio of 0.57 (95% CI: 0.26, 1.28) of clinical influenza while those in the face mask and hand hygiene group had an estimated odds ratio of 0.76 (95% CI: 0.34, 1.70), compared to the control group. We anticipate that the continued recruitment of a further 300 households by September 2008 will provide adequate power for detecting moderate effect sizes of face

masks and/or hand hygiene. The final dataset including virologic outcomes will be available by the date of the conference.

## Reference:

- [1] Cowling B.J., Fung R.O.P., Cheng K.Y., Fang V.J., Chan K.H., Seto W.H., Yung R., Chiu B., Lee P., Uyeki T.M., Houck P.M., Peiris J.S.M., Leung G.M. Preliminary findings of a randomized trial of non-pharmaceutical interventions to prevent influenza transmission in households. PLoS ONE, 2008 (in press).

### 3 VIRUS STRUCTURE AND REPLICATION

3-001

#### Improvement of Influenza B Virus Growth Phenotype by Specific Amino Acid Substitutions in Hemagglutinin

Lugovtsev, Vladimir<sup>1</sup>; Vodeiko, G.<sup>2</sup>; Levandowski, R.<sup>2</sup>; Weir, J.<sup>2</sup>

<sup>1</sup>Center for Biologics Evaluation and Research, Food and Drug Administration, USA; <sup>2</sup>CBER/FDA, USA

Adaptation of influenza B viruses to embryonated chicken eggs, a substrate for influenza vaccine production, often associates with fine modifications of the viral hemagglutinin (HA) around the receptor binding domain. Although, such a modification is beneficial for virus replication, it often leads to an alteration of antigenic properties. Serial passage in eggs of strain B/Victoria/504/2000 selected a high growth variant which acquired three amino acid (AA) substitutions in HA, G141E, R162M and D196Y. Using reverse genetics technology, we generated virus variants with different combinations of these AA substitutions. Analysis of the viral variants with single AA substitutions revealed that each of the three AA changes had a different effect on the viral phenotype. Substitution R162M was found to be most favourable, rendering the virus a high growth phenotype without antigenic alteration. Although the change G141E also resulted in high growth, it affected the antigenic properties as well. In contrast, substitution D196Y had the least effect on the virus growth and was associated with a significant antigenic deviation. The AA substitutions advantageous for growth capabilities of B/Victoria/504/2000 had similar effects when introduced into the HA of B/Beijing/184/93. In addition, we found that viral growth capability can be improved by specific AA substitutions at positions 126, 129 or 137. Although most of the AA changes analyzed in this study did affect the receptor binding properties, no correlation was observed between growth characteristics and binding profiles. This study demonstrates that modification of viral HA by carefully selected AA substitutions can be used to improve viral growth capabilities preserving the antigenic properties.

3-002

#### Elucidating the mechanism of influenza A mRNA export from the nucleus

Read, Eliot; Digard, P.

University of Cambridge, UK

Eliot Read and Paul Digard. Division of Virology, Department of Pathology, University of Cambridge, Tennis Court Road, CB2 1QP. Influenza A virus synthesises capped and polyadenylated mRNAs in the nuclei of infected cells. Although structurally indistinguishable from cellular mRNAs, they are synthesised by the viral RNA dependent RNA polymerase rather than host RNA Pol II. In addition, the viral mRNA polyA tail is produced via stuttering on a polyU tract of the genomic vRNA as opposed to the cellular polyA synthesis machinery. Furthermore, most do not contain introns, and of the two that do, the majority of the primary transcripts are not spliced. This poses questions regarding the mechanism(s) by which they are exported to the cytoplasm, as trafficking of cellular mRNAs is generally dependent on full processing of the pre-mRNA, which is co-transcriptionally linked to Pol II activity. Previously, we have shown that export of unspliced viral late gene M1 mRNA but not the early class NP transcript is dependent on continued Pol II transcription (Amorim *et al.*, 2007), suggesting that the former viral mRNA is fed into a cellular mRNA export pathway. Current evidence suggests that the bulk cellular mRNA export factor, TAP, is not involved (Satterly *et al.*, 2007) but no alternative export route has been identified. In further characterisation of the export pathways used by influenza virus mRNAs, we have determined that most viral mRNAs require Pol II activity for their export. This includes the early class intronless polymerase gene mRNAs and the intron-containing but unspliced NS1 transcript, with only the NP mRNA being predominantly cytoplasmic in the presence of the pol II inhibitor DRB. The kinetics of NP mRNA synthesis are not sufficiently different from the other viral mRNAs to explain this difference, raising the possibility that the former utilises a distinct export pathway. However, the insertion of influenza virus sequences into an HIV-1 derived Rev-responsive mRNA reporter system provided no evidence for a functional Constitutive Transport Element (CTE) able to override splicing of the chimaeric mRNAs in any influenza mRNA. Nevertheless, expression of viral mRNAs from a transfected recombinant influenza mini-genome system indicates that no viral proteins other than the polymerase and NP are required for export of viral mRNAs of segments 4 (HA) and 8 (NS1). However, segment 7 (M1) mRNAs show both predominantly nuclear and predominantly cytoplasmic mRNA in the transfected cells. These results are consistent with the possibility of distinct export pathways for the mRNAs of different



segments. Nonetheless, consistent with current evidence, our data suggests that TAP is not involved in influenza mRNA export and neither is the alternative CRM1 mediated export pathway. Work is therefore ongoing to identify the pathways used, with attention currently focussed on the role of the poly(A) tail.

Amorim, M., Read E., *et al.* (2007). "Nuclear export of influenza A virus mRNAs requires ongoing RNA polymerase II activity." *Traffic*: 1-11.

Satterly, N., Tsai P., *et al.* (2007). "Influenza virus targets the mRNA export machinery and the nuclear pore complex." *PNAS* 104(6): 1853-1858.

3-003



## Annexin II incorporated into influenza virus particles supports virus replication by converting plasminogen into plasmin

Riteau, Beatrice<sup>1</sup>; Lebouder, F<sup>2</sup>; Morello, E<sup>2</sup>; Rimmelzwaan, G.F<sup>3</sup>; Riteau, B.<sup>2</sup>

<sup>1</sup>INRA; <sup>2</sup>INRA, France; <sup>3</sup>Rotterdam, Netherlands

For influenza viruses to become infectious, proteolytic cleavage of the hemagglutinin is essential. This is usually mediated by trypsin-like proteases in the respiratory tract. Binding of plasminogen to influenza virus A/WSN/33 leads to cleavage of HA, a feature determining its pathogenicity and neurotropism. Here, we demonstrate that plasminogen also promotes the replication of additional influenza virus strains. Inhibition of the conversion of plasminogen into plasmin blocks the viral replication. Evidence is provided that activation of plasminogen is mediated by a host cellular protein, annexin-2, which is selectively incorporated into the virus particles. Indeed, inhibition of plasminogen binding to annexin-2, using a competitive inhibitor, inhibits plasminogen activation in plasmin. Altogether these results suggest that the annexin-2-mediated activation of plasminogen supports the replication of influenza viruses and contributes to their pathogenicity.

3-005

## Genes coding for polymerase proteins are essential for attenuation of Russian master donor strains for both A and B live cold-adapted reassortant vaccine

Kiseleva, Irina<sup>1</sup>; Larionova, N.<sup>1</sup>; Voeten, J.T.M.<sup>2</sup>; Teley, L.C.P.<sup>2</sup>; Drieszen-van der Cruysen, S.K.M.<sup>2</sup>; Heldens, J.G.M.<sup>2</sup>; van den Bosch, J.F.<sup>2</sup>; Rudenko, L.<sup>1</sup>

<sup>1</sup>Institute of Experimental Medicine RAMS, Russian Federation; <sup>2</sup>Nobilon International B.V., Netherlands

**Background:** The cold-adapted (*ca*) A/Leningrad/134/17/57 (H2N2) (MDV-A) and the *ca* B/USSR/60/69 (MDV-B) influenza viruses were derived from wild-type parental viruses after serial passages in eggs at 25°C and are currently in use for preparing Russian trivalent live attenuated (*att*) reassortant influenza vaccine. The MDV based live influenza vaccine's attenuated properties, temperature sensitivity (*ts*) and ability to grow at a low temperature of 25°C are inherited from the six internal genes of the MDV.

**Methods:** In this study 178 reassortants of *ca/ts/att* MDV-A with current *non-ts* influenza A viruses and 33 reassortants of *ca/ts/att* MDV-B with current *non-ts* influenza B viruses were produced in eggs or MDCK cells and were evaluated in order to identify the genes responsible for their *ts* phenotype. The genome composition of reassortants was monitored by RFLP analysis or mix PCR. *ts* phenotype was determined by titration of the reassortants in eggs or MDCK cells at optimal (32°C) and restrictive (37-40°C) temperatures and expressed as EID<sub>50</sub> or TCID<sub>50</sub>.

**Results:** Reassortants that inherited at least two polymerase genes (PB2 and PA or PB1) from MDV-A regularly demonstrated the *ts* phenotype. Reassortants that inherited PA and/or PB1 polymerase genes from the MDV-A had *non-ts* phenotype comparable to that of the wild-type viruses. The polymerase PA and PB2 gene segments of MDV-B each controlled manifestation of the *ts* phenotype of MDV-B based reassortants. The other genes coding internal proteins played no role in manifestation of the *ts* phenotype of MDV-A and MDV-B.

**Conclusion:** These data suggest that mutations in the PB2 and PB1 genes play a critical role in the temperature sensitivity of the MDV-A. Mutations in the PB2 and PA genes are responsible for the *ts* phenotype of the MDV-B. Since the *ts* phenotype is essential for attenuation, our results suggest that mutated polymerase genes play an essential role in attenuation of Russian MDV-based influenza A and B reassortant vaccine viruses.



### Studying the nuclear dynamics of the influenza A virus polymerase using fluorescence recovery after photobleaching (FRAP)

Foeglein, Á.<sup>1</sup>; Kreysa, E.<sup>1</sup>; von-Kirchbach, J.<sup>2</sup>; Digard, P.<sup>1</sup>

<sup>1</sup>Division of Virology, Department of Pathology, University of Cambridge, UK;

<sup>2</sup>DAMTP, Centre for Mathematical Sciences, University of Cambridge, UK

**Background:** The PA, PB1 and PB2 polymerase proteins of Influenza A virus form a complex in the nucleus of infected cells where, in concert with viral NP, they transcribe and replicate the viral genome. In order to achieve this, the virus has to interact with various host cell macromolecules, including components of the cellular transcription machinery. For example, PB2 interacts with pre-mRNA cap structures while the viral polymerase complex as a whole binds to cellular RNA polymerase II. Such virus-host interactions may also play a role in determining species specificity. To better understand these processes, we have developed a system for measuring the dynamics of the Influenza A virus polymerase in mammalian and avian cells to investigate the effect of various factors on the dynamics of the complex in live cells.

**Methods:** Single components or combinations of WT or mutant derivatives of the viral polymerase were transfected into mammalian 293T or avian DF1 cells, where one component was marked with a GFP-tag. The mobility of the individual components and the various complexes was subsequently measured (by calculation of diffusion coefficients and times to half recovery) by fluorescence recovery after photobleaching (FRAP). Western blots were used to confirm expression of all the transfected components. To test for replicational/transcriptional competence of the polymerase complexes, minireplicon assays containing NP and a poll-promoter driven luciferase-reporter pseudo-segment were used.

**Results:** The mobility of the full trimeric polymerase complex was significantly slower than that of any of the individual subunits or dimers, indicating that it is hindered from free diffusion. This reduction in mobility is plausibly due to interactions with cellular proteins, as the increase in size from a polymerase monomer or dimer to the trimeric complex is not sufficient to account for the decrease alone.

We hypothesised that the slow mobility of the viral polymerase complex might result from interactions with the cellular transcription machinery. In support of this, inhibition of RNA Pol II by actinomycin D or its degradation by  $\alpha$ -amanitin altered the mobility of the viral polymerase complex. However, preliminary results suggest that interactions with pre-mRNA cap structures are not a major influence on the dynamics of the viral polymerase complex as mutation of the PB2 cap-binding residue F404 did

not increase the diffusional mobility of the polymerase complex. Interestingly however, the mutation of glutamic acid (E) to lysine (K) at position 627 in PB2, which is described to be responsible for the adaptation from avian to mammalian host specificity in several cases, had a strong influence on polymerase mobility in the human 293-T cell. Incorporation of an avian virus PB2 with E627 into an otherwise human virus-derived polymerase complex led to a transcriptionally inactive polymerase with very slow diffusion in mammalian cells. However, mutation of residue 627 to lysine restored normal mobility and activity of the polymerase complex. Experiments are in progress to further define the role of PB2 627 in this system and to analyse the contribution other functional attributes of the polymerase (such as endonuclease activity and oligomerisation) make to the dynamics of the complex.

**Conclusions:** Various factors have an influence on the dynamics of the viral polymerase in the nucleus. Pol II activity proved to be relevant for the dynamics of viral polymerase, but not necessarily through a supply of capped pre-mRNA primers. Furthermore, the PB2 627 host range polymorphism negatively influenced polymerase mobility in mammalian cells. This suggests the hypothesis that it may influence polymerase function in mammalian cells by a dynamic interaction with a relatively static cellular component.

3-007



### Determinants of enhanced replication of human H7N7 influenza A virus isolates

de Wit, Emmie; Munster, V.J.; van Riel, D.; Beyer, W.E.P.; Rimmelzwaan, G.F.; Kuiken, T.; Osterhaus, A.D.M.; Fouchier, R.A.M.

Department of Virology, Erasmus MC, Netherlands

In 2003, an outbreak of highly pathogenic avian influenza (HPAI) virus (H7N7) occurred in the Netherlands, during which 89 humans were infected. Most persons suffered from conjunctivitis and, occasionally, mild respiratory disease. There was one fatal case of pneumonia. The virus isolated from the fatal case (FC) differed in 15 amino acid positions in five gene segments from a prototype conjunctivitis case (CC) virus. In a mouse model, we have shown that the E627K substitution in PB2 of the FC virus was the main determinant of pathogenicity in these animals. Here, we show that each of the five gene segments that harbored amino acid substitutions contributed to increased virus replication efficiency in mammalian and avian cells in vitro. In minigenome assays, PB2 of the FC virus caused a large increase in reporter expression levels. This increase was caused by the E627K substitution in PB2 of the FC virus and was independent of temperature and host cell. The PA of the FC virus had an additive

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effect on reporter expression levels in the context of the FC virus polymerase complex. The HA gene of the FC virus resulted in increased virus replication in reassortant viruses. This effect was caused by a single amino acid substitution, A143T, which also affected the attachment pattern of the FC virus to tissues of the human respiratory tract. The inefficient rescue of viruses with NA of the CC virus indicated that NA had a large effect on virus replication in vitro. Site-directed mutagenesis indicated that multiple substitutions were responsible for the NA phenotype. We did not observe differences in the interferon-antagonism function of FC and CC NS1 genes. We conclude that the pathogenic properties of the FC virus could be determined by genetic changes in at least four genes that affect virus replication in vitro. Detailed analyses of mutations that facilitate replication of avian viruses in mammalian cells remain important for assessing the risks posed by such zoonotic viruses.

3-008

### The second sialic acid binding site of influenza virus NA serves to enhance its catalytic efficiency

**Uhlendorff, J.;** Matrosovich, T.; Klenk, H.D.; Matrosovich, M.

*Institute of Virology, Philipps University, Marburg, Germany*

Neuraminidases (NA) of avian influenza viruses possess, in addition to the catalytic site, a second sialic acid binding site that displays hemadsorption (HAD) activity. In contrast, NAs of human and swine influenza viruses show weak if any HAD activity. Neither the biological function of the HAD site, nor reasons for its loss in human viruses are known. To address these questions, we cloned the NA of 1957 pandemic virus A/Singapore/1/57 (H2N2) that differs from the avian ancestor NA by a single amino acid substitution in the HAD site (S367N). We found that SG/57 NA lacks HAD activity and was able to restore this activity by mutating Asn at position 367 back to the avian-virus-like Ser. Using the HAD-positive NA, we generated three additional NA variants with single-point amino acid substitutions S370L, N400S and W403R which have been found in the HAD-site of other human H2N2 isolates. Each of these mutations abolished HAD-activity. All five NA variants hydrolyzed monovalent substrates with identical efficiency. However, HAD-positive NA cleaved macromolecular substrates much faster than the HAD-negative counterparts. We conclude that the second sialic acid binding site enhances catalytic efficiency of the NA by increasing its affinity for macromolecular substrates. Our data imply that the loss of HAD activity and concomitant reduction of the NA catalytic efficiency might have been required for the emergence of the 1957 pandemic virus.

3-009

### The influenza B virus accessory protein NB mediates efficient spread of virus through the airways

**Barclay, W.<sup>1</sup>;** Elderfield, R.A.<sup>1</sup>; Jackson, D.<sup>2</sup>; Roberts, K.<sup>1</sup>; Hennessey, M.<sup>3</sup>; Pickles, R.<sup>3</sup>

*<sup>1</sup>Imperial College, UK; <sup>2</sup>University of Reading, UK; <sup>3</sup>University of North Carolina, USA*

In addition to the viral neuraminidase, all isolates of influenza B virus encode a second protein from RNA segment 6, termed NB. The NB protein is a 100 amino acid, type III transmembrane protein with two glycosylation sites in its ectodomain that are modified in cells to bear polylactosaminoglycan carbohydrate chains. Using a reverse genetic approach, we have generated isogenic viruses based on the strain B/Beijing/87 that differ only in the NB protein. Here we show that despite equal replication in polarized MDCK cells, viruses that either lack NB protein altogether, or encode NB that is not glycosylated, are severely compromised in their replication in differentiated ciliated airway epithelial cultures of human or ferret origin. In the absence of glycosylated NB, virus replicated to  $3.5 \times 10^4$  pfu/ml compared to  $3.3 \times 10^6$  pfu/ml for wild-type virus and exhibited a reduction in spread through airway cultures. Accordingly, replication in the ferret respiratory tract in vivo was reduced at least 10-fold for NB mutant viruses, with peak viral titres occurring 4 days later than observed for the wild-type virus in nasal washes of infected animals. Since in the absence of glycosylation, NB is poorly transported to the cell surface and less NB protein is incorporated into virions, these data suggest an important role for virion NB in facilitating cell to cell spread through the carbohydrate-rich airway surface microenvironment of the ciliated respiratory epithelium.

3-010

### Activation and neutralization of PKR by influenza B virus: evidence for a crucial NS1-PKR complex that is bridged by dsRNA

**Wolff, T.W.<sup>1</sup>;** Schneider, J.S.<sup>1</sup>; Waibler, Z.W.<sup>2</sup>; Kalinke, U.K.<sup>3</sup>; Dauber, B.D.<sup>1</sup>

*<sup>1</sup>Robert Koch-Institute, Germany; <sup>2</sup>Paul Ehrlich-Institut, Germany; <sup>3</sup>Paul Ehrlich-Institute, Germany*

The IFN-inducible kinase activated by dsRNA (PKR) is a major component of the innate antiviral response. Activation of this dsRNA-dependent enzyme results in the phosphorylation of the translation initiation factor eIF2 $\alpha$ , which blocks protein synthesis and thereby impairs virus replication. We previously

demonstrated that the dsRNA binding NS1 protein of influenza B virus prevents activation of PKR (Dauber *et al.*, 2006; J Virol 80). Here we determine that this NS1 activity is crucial for efficient viral propagation and also describe a novel model that explains the mechanism of this viral countermeasure.

Engineered influenza viruses expressing dsRNA binding-deficient NS1 proteins were strongly attenuated for growth on embryonic fibroblasts of wild-type mice. However, this growth phenotype was largely rescued on PKR  $-/-$  cells indicating that PKR is the primary target of NS1's dsRNA-binding activity.

Activation of PKR has previously been suggested to occur by dsRNA-intermediates of viral genome replication, but little evidence has been presented for this model concerning influenza viruses. In fact, a kinetic analysis revealed that the timing of PKR activation did not coincide with the early replicative phase, but was rather associated with the cytosolic appearance of viral ribonucleoprotein (RNP) late in virus infection. Furthermore, in vitro kinase studies showed that purified viral RNP is a potent activator of PKR indicating that this can also happen inside cells. Significantly, co-immunoprecipitation and pull-down analyses revealed that the viral NS1 protein forms a physical complex with PKR in infected cells. This interaction was sensitive to double strand-specific RNase and was not detected when RNA binding was inactivated by mutation in either the viral NS1 protein or PKR. Further studies showed that PKR co-sediments with the viral NS1 and NP proteins upon sucrose gradient fractionation of cell lysates. We therefore propose a new model in which PKR is activated by a double-stranded structure contained in the viral RNP, with the viral NS1 protein via an RNA-mediated interaction inhibiting this induction.

3-011

### Determinants of viability of influenza A viruses harboring an NS segment with heterotypic type C non-coding sequences

Crescenzo-Chaigne, B.; Frigard, V.; van der Werf, S.

Institut Pasteur, France

Type C viral like RNAs have been shown to be transcribed/replicated by the type A polymerase whereas the type C polymerase is not able to transcribe/replicate a type A viral RNA template efficiently. It has been shown previously that the nature of nucleotides 5 at the 3' end, 6' at the 5' end and of base pairing between nucleotides 3' and 8' at the 5' end are important determinants of this type specificity. In this study we analyzed the effects of substitution of the 5' and/or 3' non-coding sequences of the NS segment of the A/WSN/33 virus by that of the NS segment of the C/Johannesburg/1/66 virus. Transient transcription/replication experiments were performed in the presence of the

type A polymerase using NS-like RNA templates harboring a CAT reporter gene sequence. It was found that substitution of the 3' non-coding sequence of type A by that of type C had no effect on the efficiency of transcription/replication of the NS-like RNA template, whereas it was reduced about 20-fold when substituting the 5' non-coding sequence. Substitution of both the 5' and 3' non-coding sequences by that of type C resulted in a 6-fold reduction of the efficiency of transcription/replication by the type A polymerase. When attempting by reverse genetics to produce type A viruses with the corresponding NS segments, no virus could be rescued with the type C 5' non-coding region either alone or in combination with the type C 3' non-coding region. Substitution of the 3' non-coding region by that of type C resulted in the production of a viable virus. Sequence analysis of the extremities showed however that nucleotide 5 at the 3' end had mutated from a C as for type C to a U residue as for type A. A systematic reversion was also observed when attempting to rescue a type A virus with a U5C change in the 3' end of the NS segment, providing strong evidence that a U residue at position 5 at the 3' end of the NS segment is essential for type A virus replication. In contrast, a type A virus in which the 3'U:8'A base pair at the 5' end was substituted by the type C like 3'C:8'G base pair could be rescued albeit with reduced efficiency as compared to wild-type and was found to retain the mutations upon further passage. Most interestingly, a type A virus with an NS segment harboring both 5' and 3' non-coding sequences from type C but retaining a type A like double panhandle at the extremities, i.e. with 3'U:8'A, 6'A and 5U residues at the 5' and 3' ends respectively, was rescued with similar efficiency as wild-type. Kinetics of virus multiplication at low multiplicity of infection (0.001) showed that this virus replicated slower than wild-type but with similar kinetics as the type A virus with a type C like 3'C:8'G base pair, whereas the type A virus with a type C 3' non-coding sequence with a C to U reversion at residue 5 replicated even slower. However, quite similar final viral titers were reached for all viruses. The nature of nucleotide 5 at the 3' end of the NS segment appeared to be crucial for the viability of a type A virus whether in the context of a type A or type C 3' non-coding sequence but not for transcription/replication process. Furthermore, the possibility to replace the type A non-coding regions of the NS segment by type C sequences that differ both in length and sequence provided the double panhandle structure at the extremities is maintained suggests that important signals for transcription/replication, intracellular trafficking as well as for packaging of the NS segment are either exclusively located in the double panhandle structure or conserved to some extent within the more distal non-coding sequences between type A and C influenza viruses.

## 4 VACCINES: CURRENT AND NOVEL APPROACHES

4-001

### The safety, immunogenicity and protective efficacy of a PER.C6® cell grown influenza H7N1 virus vaccine in preclinical and clinical studies

**Cox, R.J.<sup>1</sup>; Major, D.<sup>2</sup>; Madhun, A.S.<sup>1</sup>; Hauge, S.<sup>1</sup>; Sjørnsen, H.<sup>1</sup>; Hoschler, K.<sup>3</sup>; Vogel, F.<sup>4</sup>; Barclay, W.<sup>5</sup>; Zambon, M.<sup>3</sup>; Wood, J.<sup>2</sup>; Campitelli, L.<sup>6</sup>; Haaheim, L.R.<sup>1</sup>**

<sup>1</sup>University of Bergen, Norway; <sup>2</sup>NIBSC, UK; <sup>3</sup>HPA, UK; <sup>4</sup>sanofi pasteur, France; <sup>5</sup>Imperial College, UK; <sup>6</sup>ISI, Italy

Highly pathogenic influenza H5 and H7 subtypes have caused outbreaks in poultry and occasionally crossed the species barrier resulting in human disease and fatalities. Preclinical and clinical work on these subtypes as vaccine candidates is necessary to allow rapid production of an appropriate vaccine in the event of a pandemic and thus increase global pandemic preparedness. The FLUPAN consortium has developed the first human vaccine against avian influenza H7 virus, which has undergone preclinical and clinical evaluation. The candidate virus (A/Chicken/Italy/13474/99 (H7N1)) was chosen from strains of highly pathogenic H7N1 avian influenza virus, which caused a lethal poultry outbreak in Italy in 1999. The vaccine strain (RD-3) was produced by reverse genetics and an inactivated split virus vaccine was produced on the PER.C6 human cell line. The vaccine was administered as non adjuvanted or aluminium hydroxide adjuvanted vaccine, which was formulated directly before administration. Preclinical studies were conducted in mice and ferrets to evaluate the immunogenicity and protective efficacy of this vaccine. Animals were immunised with two doses of vaccine approximately three weeks apart. Balb/c mice were immunised subcutaneously with 12 or 20 µg haemagglutinin (HA) per dose with or without aluminium hydroxide adjuvant. Ferrets were vaccinated intramuscularly with 24µg HA of the adjuvanted vaccine. Animals were challenged after the second immunisation with the parent highly pathogenic influenza H7N1 virus. Clinical signs, virus replication and survival were recorded. The vaccine induced low haemagglutination inhibition (HI) antibody responses after two doses of vaccine, however vaccinated animals had a significantly higher survival rate, and significantly lower weight loss and viral replication than control animals. A phase I clinical trial was conducted in 60 healthy volunteers (21 males, 39 females) to evaluate the safety and immunogenicity of this vaccine. Volunteers were randomly assigned to one of four groups containing 15 subjects, which received two doses of the H7N1 virus vaccine containing 12µg or 24µg HA formulated with aluminium hydroxide adjuvant (300µg or 600µg, respectively) or administered without adjuvant. Five volunteers received only one dose of vaccine. Blood samples were collected at frequent

time intervals to evaluate the kinetics of the influenza specific cellular and serum antibody responses. In humans, the vaccine was well tolerated and no serious adverse events were reported. Local and systemic reactions were usually mild and transient resolving within three days of immunisation. Pain at the injection site was more frequently observed in the adjuvant groups than in the non adjuvant groups. Twenty-one of fifty-four volunteers had low antibody responses after the second vaccination detected by either HI or microneutralisation assays. Formulation with aluminium adjuvant significantly enhanced the number of responders irrespective of vaccine strength (8 of 13 volunteers in the 24 µg HA adjuvanted vaccine group and 7 of 14 volunteers in the 12 µg HA adjuvanted group compared to 3 volunteers in each of the non-adjuvanted 12 µg and 24 µg HA vaccine groups). The antibody secreting cell and serum antibody responses were dominated by IgG and IgA, with almost no IgM response detected. This unexpected absence of IgM requires further investigation. Importantly in preclinical animal models, the vaccine also elicited low antibody responses but nevertheless provided significant protection from clinical illness and/or death after a challenge with the highly pathogenic parent virus. Our findings raise concerns about the criteria used by the Committee for Medicinal Products for Human Use (CHMP) for assessing surrogate correlates of protection to candidate pandemic vaccines. There is therefore an urgent need for more research to allow better understanding of mechanisms of protection against avian influenza viruses.

4-002

### Induction of protective immunity after a single vaccination with an inactivated cell culture derived whole virus pandemic influenza vaccine adjuvanted with CoVaccine HT.

**Glansbeek, Harrie; Bruijini de, M.A.M.; Heldens, J.G.M.; Hilgers, L.A.T.; Bosch van den, J.F.**

*Nobilon Schering-Plough, Netherlands*

Control of an influenza pandemic by vaccination depends on availability and efficacy of a vaccine. Currently, most pandemic influenza vaccines are adjuvanted with aluminium salts. Results from different trials in humans indicate that two vaccinations with aluminium adjuvanted vaccines are required to induce sufficient protective immunity. A vaccine that induces protection after a single vaccination with a low antigen dose would have major advantages with respect to the onset of immunity, antigen sparing, and logistics of large vaccination campaigns. In an attempt to improve the immunogenicity of an inactivated cell culture derived whole H5N1 virus influenza vaccine, the effects of the adjuvants aluminium hydroxide and CoVaccine HT were compared in animal models. CoVaccine HT contains a

sucrose fatty acid sulphate ester incorporated in a submicron squalane-in-water emulsion.

Vaccination studies in ferrets showed that aluminium hydroxide clearly enhanced the hemagglutination inhibition (HI) titers against the vaccine strain. Co-delivery of CoVaccine HT appeared to be a much stronger adjuvant as the HI titers after two vaccinations were more than 8-fold higher as compared with aluminium hydroxide. Interestingly, 3 weeks after a single vaccination of CoVaccine HT-adjuvanted pandemic influenza vaccine (7.5 µg HA/dose, strain NIBRG14), the geometric mean HI titre (GMT) was 1:294 while aluminium-adjuvanted vaccine induced a GMT of 1:34. As a GMT of 1:40 is considered to be protective, this result indicates that protective immunity was induced after a single vaccination with a CoVaccine HT-adjuvanted pandemic influenza vaccine containing a low dose of antigen. In addition to the ferret data, the results of a vaccination trial in cynomolgus macaques will be presented.

Because T-cells recognize epitopes from conserved antigens, such as the M and NP proteins, which are not accessible to antibodies, the induction of cell-mediated immunity might be important for maximizing the induction of cross-protective immunity. To study the type of immune response, vaccination studies were performed in mice. Mice were vaccinated with aluminium hydroxide adjuvanted or CoVaccine HT-adjuvanted vaccines containing inactivated whole virus (strain A/Pr/8/34). Based on IgG1/IgG2a ratios, it was demonstrated that the aluminium-hydroxide adjuvanted vaccine induced a biased Th2 response while co-delivery of CoVaccine HT induced a more balanced Th1/Th2 response, which indicates cell-mediated immunity.

In summary, CoVaccine HT induces a mixed Th1/Th2 response in mice and high HI titers after a single vaccination in ferrets. These results indicate that the adjuvant CoVaccine HT is a promising adjuvant for the development of a single-shot pandemic influenza vaccine.

4-003

#### Quantitation of haemagglutinin in H5N1 influenza viruses reveals low haemagglutinin content of vaccine virus NIBRG-14 (H5N1)

**Harvey, Ruth;** Wheeler, J.X.; Wallis, C.L.; Robertson, J.S.; Engelhardt, O.G.

National Institute for Biological Standards and Control, UK

The assessment of potential influenza virus vaccine strains includes a number of factors. Growth properties of the virus and yield of antigen, specifically the haemagglutinin (HA), are of key importance. The recently developed H5N1 vaccine reference strain NIBRG-14 (with HA and NA genes derived from the clade 1 virus

A/Viet Nam/1194/2004 in an A/Puerto Rico/8/34 background) has been suggested to yield low amounts of antigen. While investigating the antigen yield of H5N1 vaccine viruses, we found that accurate quantitation of the HA content of some H5N1 viruses was difficult due to the migration characteristics of the proteins on SDS PAGE gels. The HA1 and HA2 bands co-migrated with nucleoprotein (NP) and matrix protein (M1) respectively, preventing accurate analysis. We have developed an accurate way of quantitating HA from these H5N1 viruses by introducing a deglycosylation step to the standard protocol. Using this method, we showed reproducibly that the low yield of NIBRG-14 is, at least in part, due to a lower than usual content of HA in virus preparations. This was also found to be the case for the parent wild-type A/Viet Nam/1194/2004 virus.

4-004

#### Influenza vaccine strain generation via reverse genetics on PER.C6® cells

**Koudstaal, W.<sup>1</sup>;** Hartgroves, L.<sup>2</sup>; Meester-Rood, P.<sup>1</sup>; Custers, J.<sup>1</sup>; Wanningen, P.<sup>1</sup>; Provacia, L.<sup>1</sup>; Ophorst, C.<sup>1</sup>; Sieuwerts, M.<sup>1</sup>; Zijldgeest, D.<sup>1</sup>; Vogels, R.<sup>1</sup>; de Boer-Luijze, E.<sup>3</sup>; Cornelissen, L.<sup>3</sup>; Legastelois, I.<sup>4</sup>; Goudsmit, J.<sup>1</sup>; Havenga, M.<sup>1</sup>; Barclay, W.<sup>2</sup>

<sup>1</sup>Crucell, Netherlands; <sup>2</sup>Imperial College, UK; <sup>3</sup>Animal Sciences Group, Netherlands; <sup>4</sup>sanofi pasteur, France

Reverse genetics allows for the generation of influenza viruses entirely from cDNA, thereby potentially presenting a fast method for the creation of vaccine strains. Reverse genetics necessitates the use of cultured cells. Due to stringent technical and regulatory requirements, the choice of cell lines for the production of human influenza vaccines is currently limited. PER.C6® cells, among the most extensively characterized and documented cells, support growth of all influenza viruses tested to date, and can be grown to high densities up to 20,000 litres in the absence of serum or micro carriers. Here, the suitability of PER.C6® cells for the generation of influenza vaccine strains by reverse genetics was investigated. We demonstrate that various influenza A strains, one influenza B strain and a wide range of 6:2 reassortants based on the A/Puerto Rico/8/34 strain could be rescued from PER.C6® cells successfully, using a variety of transfection methods, including serum-free procedures. Furthermore, we assessed the protective efficacy of a vaccine based on a reassortant carrying the HA and NA segments of A/HK/156/97 that was both rescued from and propagated on PER.C6® cells in a stringent mouse model. Based on the data obtained thus far we conclude that PER.C6® cells provide a robust platform for the generation of influenza vaccine strains via reverse genetics.

4-005

# Induction of cross-clade anti-H5N1 immune responses in mice, guinea pigs and ferrets by Vero cell-derived H5N1 whole virus candidate vaccines

**Kistner, Otfried**; Howard, M.K.; Sabarth, N.; van Maurik, A.; Wodal, W.; Kerschbaum, A.; Grillberger, L.; Tauer, C.; Reiter, M.; Mundt, W.; Livey, I.; Barrett, P.N.

Baxter Innovations GmbH, Austria

The re-emergence of highly pathogenic avian influenza viruses (HPAI) of subtype H5N1 in South East Asia since 2003, their spread over several African and European countries by migratory birds, and the infection of more than 370 humans with a case-fatality-rate of about 60% has increased the awareness of the threat of a new influenza pandemic. This has resulted in pandemic preparedness activities including the development and production of H5N1 vaccines for either stockpiling of currently available candidate vaccines or advanced purchase agreements of vaccines against newly emerging pandemic influenza viruses. Both approaches have their specific advantages and disadvantages; stockpiling of the vaccine ensures its immediate availability whenever a pandemic starts, but it may be less effective because of antigenic mismatches with the actual pandemic strain. On the other hand, the production of a pandemic vaccine in the scope of advanced purchase agreements can result in a more effective vaccine by using the actual pandemic virus, however, huge quantities of vaccine have to be produced within a short timeframe.

Baxter has produced H5N1 whole virus candidate vaccines against H5N1 clade 1 (A/Vietnam/1203/2004) and clade 2 (A/Indonesia/05/2005) strains at an industrial scale using its serum protein free Vero cell fermenter technology. These candidate vaccines have been shown to be highly immunogenic in mice and guinea pigs by inducing cross-neutralizing antibodies (which has been confirmed in clinical phase I/II and III studies in humans) and showing cross-protection against the homologous strain of the same clade and the heterologous strains of other clades in mice. The protective efficacy of the vaccine at doses as low as 3.75µg has been confirmed in ferret challenge studies.

The immunization and protection studies in guinea pigs and mice have been extended by performing heterologous prime-boost studies i.e. priming with the clade 1 Vietnam 1203 candidate vaccine followed by a booster with the clade 2 Indonesia candidate vaccine or vice-versa. These studies have shown that the heterologous prime-boost strategy resulted in broad anti-H5N1 immune responses with excellent cross-neutralization activities and cross-protectivity against representative H5N1 viruses of clade 1, clade 2, subclades 1 and 2, as well as clade 0 strains.

The impact of these results on preparedness strategies will be discussed.

4-006

# A probiotic fermented dairy product improves immune response to influenza vaccination in elderly people

**Vaudaine, Sarah**<sup>1</sup>; Aubin J-T<sup>2</sup>, Boge T<sup>4</sup>, Remigy M<sup>3</sup>, Tanguy J<sup>1</sup>, Borgies B<sup>1</sup>, Tondou F<sup>1</sup>, Bourdet-Sicard R<sup>1</sup>, van der Werf S<sup>2</sup>, Samson SI<sup>1</sup>.

<sup>1</sup>Danone Research, France; <sup>2</sup>Unit of Molecular and Genetic of Respiratory Viruses, Pasteur Institute, 25 rue du Dr Roux, Paris (France); <sup>3</sup>Geriatrician, Maison de retraite Sainte Famille, Metz (France); <sup>4</sup> Geriatrician, Avenue Louis Jourdan, Bourg en Bresse (France)

The influenza viruses are a major cause of respiratory infections. Winter influenza epidemics affect 1 to 5% of the population and is major public health concern especially for the elderly for which complications may occur. It is known that the elderly respond poorly to influenza vaccination compared to younger adults. This work addresses the question whether daily consumption of a specific probiotic fermented dairy product could improve vaccination responses in the elderly.

Two randomized, controlled, double-blind, multicentric clinical trials were conducted in France during the vaccination campaigns of 2005-2006 and 2006-2007. The subjects consumed daily a specific fermented dairy product, Actimel®, containing the probiotic strain *Lactobacillus casei* DN-114 001 or a control product during 7 weeks (pilot study), or 13 weeks (confirmatory study). Consumption of the study products started 4 weeks prior to vaccination. The specific antibody levels were measured against the three influenza viral strains composing the vaccine (H1N1, H3N2 and B), before and after vaccination, by haemagglutination inhibition (HI) test. Antibody geometric mean titres, changes in seroprotection (antibody titre equal to 1:40 in the HI test) and changes in seroconversion (4-fold increase in antibody titres after vaccination) were compared between the two groups under study.

In the pilot study conducted on 86 elderly subjects, influenza-specific antibody titres were increased in the probiotic group 3 weeks after vaccination but the difference did not reach statistical significance compared to the control group. A statistical trend (p=0.080) in favour of the probiotic group for seroprotection towards the H1N1 strain was identified (64.0% for probiotic versus 42.5% for the control group). Consumption of the probiotic drink was stopped 3 weeks after vaccination and, 3 months after vaccination, antibody titres against the 3 viral strains were back to baseline levels. This exploratory study suggested that consumption of a probiotic dairy product can improve antibody responses to influenza vaccination.

A confirmatory clinical study was conducted on the next vaccination campaign and was designed with the appropriate calculation of subjects to confirm the results of the pilot study. For the 222 elderly subjects included, antibody titres of the probiotic group were higher for the three strains at 3, 6 and 9 weeks after vaccination compared to the control group. This difference was

statistically significant for the B strain at 3, 6 and 9 weeks post vaccination (respectively  $p=0.029$ ,  $p=0.027$ ,  $p=0.026$ ). There were no significant differences for H1N1 and H3N2 strains. For the B strain, the effect of the probiotic dairy product on antibody titres was significant overtime ( $p=0.020$ ) by maintaining higher antibody levels under product consumption compared to the control. These results on the B strain were statistically confirmed regarding the seroconversion criteria. In addition, in non-seroprotected subjects at inclusion, the probiotic group showed a significant higher seroprotection 3 weeks after vaccination for the H1N1 strain ( $p=0.024$ , Chi2 test) and for the 2 Influenza A strains (H1N1 and/or H3N2,  $p=0.031$ , Chi2 test) compared to the control group. Finally, five months after vaccination - consumption of study products being stopped after 9 weeks - specific antibody titres against H1N1 and B strains were back to baseline levels in both groups. Concerning the H3N2 strain, specific antibody levels were back to baseline levels in the control group and higher in the probiotic group but not statistically different between the two groups.

In conclusion, these clinical studies provide evidence that a specific probiotic fermented dairy product can improve immune responses to influenza vaccination evaluated by specific antibody titres in the elderly population, known to have a reduced response to influenza vaccine.

4-007

#### **MF59™ adjuvanted influenza vaccine (Fluad®) in the elderly: greater immunogenicity against a heterovariant A/H3N2 strain, compared with split and virosomal vaccines**

**Baldo, V.<sup>1</sup>**; Baldovin, T.<sup>1</sup>; Pellegrini, M.<sup>2</sup>; Busetti, F.<sup>1</sup>; Groth, N.<sup>2</sup>; Angiolelli, G.<sup>3</sup>; Trivello, R.<sup>1</sup>

<sup>1</sup>Department of Environmental Medicine and Public Health, Institute of Hygiene, University of Padua, Italy; <sup>2</sup>Global Clinical Research and Development, Novartis Vaccines & Diagnostics S.r.l., Siena, Italy; <sup>3</sup>Local Health Unit n.13, Veneto Region, Italy

**Background and Aim:** Influenza A/H3N2 infection emerged in 1968, causing a pandemic, and has subsequently undergone considerable antigenic and genetic variation. A/H3N2 strains have been associated with more severe epidemics than the other currently circulating influenza viruses (A/H1N1 and B), especially in vulnerable populations, such as the elderly with underlying medical conditions. MF59™ adjuvant has been shown to enhance immunogenicity of subunit influenza vaccine for both homologous and heterologous influenza strains. Immunity against heterologous strains is of particular medical significance during influenza epidemics when mismatches between vaccine

strains and circulating influenza viruses occur. This study aimed to confirm if the presence of MF59™ in the vaccine formulation could improve immune responses against a heterovariant A/H3N2 strain in elderly subjects with chronic conditions.

**Methods:** In a randomized, double-blind trial, elderly nursing home residents ( $\geq 65$  years of age) in North-east Italy received either an MF59™ adjuvanted influenza vaccine (Sub/MF59; FLUAD®, Novartis Vaccines), a split vaccine (Split; Mutagrip®, Pasteur Merieux MSD), or a virosomal vaccine (SVV; Inflexal-V®, Swiss Serum and Vaccine Institute) during the winter season of 1998/99; the majority of subjects had at least one underlying chronic disease, including a heart or lung condition or diabetes mellitus. Study vaccines contained the strains recommended by the WHO for 1998/99 Northern Hemisphere formulation (A/H3N2/Sydney/5/97; A/H1N1/Beijing/262/95 and B/Beijing/184/93). Blood samples were obtained pre-vaccination and at 4 weeks post-vaccination. Hemagglutination inhibition (HI) titres were measured against the A/H3N2 influenza antigen recommended for the 2006/07 vaccine formulation: A/H3N2/Wisconsin/67/2005. Pre- and post-vaccination geometric mean antibody titres (GMTs), the post-vaccination mean-fold increase in HI antibodies (MFI), the number of subjects with protective HI titers ( $\geq 40$ ), and the number of subjects with a four-fold increase in post-vaccination titers were calculated. Sera from 199 subjects were available for analysis (Sub/MF59:  $n=72$ ; Split:  $n=88$ ; SVV:  $n=39$ ). More than 80% of subjects in each vaccination group were over 75 years of age. The Split group included more healthy subjects compared with the Sub/MF59 and SVV groups (39.8%, 12.5% and 20.5%, respectively). There were no male subjects in the SVV group (vs. 6.9% in the Sub/MF59 and 27.3% in the Split group).

**Results:** No statistically significant differences in baseline GMTs were observed between vaccine groups. Post-vaccination HI antibody titres against the heterovariant A/H3N2 strain were significantly higher ( $p=0.02$ ) in the Sub/MF59 group, compared with SVV and Split groups. Compared with Split and SVV vaccines, MF59™ adjuvanted vaccine resulted in higher MFI (2.0, 2.0 and 3.1, respectively), and significantly greater proportions of subjects with at least a fourfold increase in antibody titers (27.3%, 23.1% and 41.7%, respectively,  $p=0.05$ ). Seroprotective antibody levels were achieved by more vaccinees in the MF59™ adjuvanted and split vaccine groups (79.2% and 78.4%, respectively) than in the virosomal group (56.4%).

**Conclusion:** MF59™ adjuvanted influenza vaccine induced higher HI antibody levels against a heterovariant A/H3N2 strain in this population of elderly people with chronic diseases, compared with conventional split and virosomal vaccines. These results confirm superior cross-reactive immunogenicity of MF59™ adjuvanted influenza vaccines. Since A/H3N2 influenza viruses are epidemiologically highly relevant for the elderly population, the broader immunogenicity conferred by FLUAD® could be of particular clinical benefit in seasons where an antigenic mismatch occurs.

4-008

### Immunization with seasonal influenza vaccines confers protection against H5N1 infection in mice

**van Maurik, Andre;** Sabarth, N.; Howard, M.K.; Kistner, O.; Savidis-Dacho, H.; Grillberger, L.; Tauer, C.; Reiter, M.; Mundt, W.; Barrett, P.N.

Baxter Innovations GmbH, Austria

The events of recent years have highlighted the need for influenza vaccines that are broadly cross-reactive against a number of subtypes with pandemic potential. We have recently reported that Baxter's Vero cell-derived H5N1 whole virus candidate vaccines have the ability to elicit broad anti-H5N1 immune responses in both pre-clinical animal models as well as in a clinical phase I/II study in humans. We demonstrated that Baxter's H5N1 whole virus candidate vaccines have excellent cross-neutralization activities and cross-protectivity against representative H5N1 viruses of clade1, clade 2, subclades 1 and 2, as well as clade 0 strains. More recently, it was reported that cross-reactive immune responses against highly pathogenic H5N1 influenza virus could also be achieved by immunizing subjects with a trivalent seasonal influenza vaccine (EID, 2008; 14: 121-128). However, whether cross-subtype immunity could protect against infection with highly pathogenic H5N1 influenza virus could not be addressed in this study with human subjects. Therefore, the aim of the studies described here was to use the mouse challenge model to evaluate whether immunization with the seasonal influenza vaccine, as a monotherapy, or in combination with H5N1 whole virus vaccine, could confer protection against H5N1 influenza virus infection. We found that Baxter's whole virus trivalent seasonal influenza vaccine had the ability to protect mice against a lethal challenge with wild-type H5N1 virus. It was found that the protective efficacy was mainly contained within the H1N1 component of the trivalent vaccine. Importantly, two homologous immunizations were required, since a single immunization with the trivalent seasonal influenza vaccine failed to protect mice against H5N1 virus infection. We next investigated whether the trivalent seasonal influenza vaccine could be used in combination with an H5N1 whole virus vaccine. We found that the heterologous immunization regimen achieved superior efficacy compared to the homologous immunization regimen using equivalent sub-optimal doses of the H5N1 whole virus vaccine. These data indicate that partial cross-type immunity can be achieved by immunization with a trivalent seasonal influenza vaccine, which can be further boosted with a Vero cell-derived H5N1 whole virus candidate vaccine.

4-010

### Haemagglutinin quantification of influenza A and B strains in egg-based whole virus and subunit vaccines by RP-HPLC as alternative for SRID

**Kapteyn, J.C.<sup>1</sup>;** Porre, A.M.<sup>1</sup>; De Rond, E.J.P.<sup>1</sup>; Hessels, W.B.<sup>1</sup>; Slotboom, A.M.E.<sup>1</sup>; Kessen, H.<sup>2</sup>; Tijms, M.A.<sup>1</sup>

<sup>1</sup>Development Influenza, Solvay Biologicals, Netherlands; <sup>2</sup>Quality Control Influenza, Solvay Biologicals, Netherlands

The haemagglutinin (HA) content is an important specification of trivalent inactivated influenza vaccines. HA in vaccines has typically been quantified by single-radial-immunodiffusion (SRID), but recently a reversed-phase high performance liquid chromatography (RP-HPLC) method for quantification of HA has been developed, being superior in terms of sensitivity (limit of quantification), precision, range, and analytical throughput (Kapteyn *et al.*, 2006). However, development of this HPLC method was entirely focused on cell culture-based whole virus vaccines. Here, we present data demonstrating that this RP-HPLC method is also highly suitable for HA quantification of egg-based whole virus in-process vaccine samples, including egg allantoic harvest, and final formaldehyde-inactivated subunit vaccines. Moreover, the HPLC assay is suitable to quantify HA from pandemic (H5N1) influenza strains. The assay specificity also enables simultaneous quantification of HA from influenza A and B-strains in the trivalent inactivated subunit influenza vaccine Influvac®. In addition, we show data that highlights that HPLC is very powerful in the early stages of trivalent seasonal vaccine production, enabling fast and reliable viral growth studies in eggs for influenza virus strain selection in vaccine production when SRD reagents are not yet available.

4-011

### Protective efficacy of cell-culture derived, whole influenza virus vaccine in the mouse and ferret model

**Geels, Mark<sup>1</sup>;** Hagenaars, N.<sup>2</sup>; Glansbeek, H.<sup>1</sup>; Mastrobattista, E.<sup>2</sup>; De Bruijini, M.<sup>1</sup>; Heldens, J.<sup>1</sup>; Jiskoot, W.<sup>3</sup>; Van den Bosch, H.<sup>1</sup>

<sup>1</sup>Nobilon / Schering-Plough, Netherlands; <sup>2</sup>Department of Pharmaceuticals, Utrecht Institute for Pharmaceutical Sciences, Netherlands; <sup>3</sup>Division of Drug Delivery Technology, Leiden/Amsterdam Center for Drug Research, Netherlands

Traditional influenza vaccine production is based on influenza seed virus propagation in embryonated chicken eggs. The disadvantages of this are the timely supply of the required number of embryonated eggs and a production process which

is inherently vulnerable to contaminations. Cell culture-based systems are more attractive for large-scale production of influenza vaccines from an operational perspective, as they are likely to be more controllable and safe. This study aimed to compare different influenza vaccine formulations and demonstrate the protective efficacy of a cell-culture derived (CC), whole influenza virus (WIV) vaccine in mice and ferret animal models.

WIV, split, subunit and virosome vaccines were generated from a single batch of MDCK-grown, mouse-adapted influenza strain A/PR/8/34 (Nobilon). Mice were vaccinated twice and challenged. Three groups of male ferrets were vaccinated twice with trivalent CC-WIV vaccine (Nobilon, Northern Hemisphere 2007-2008 recommendation), a commercially available split influenza vaccine or a placebo and subsequently challenged with the wild-type human H3N2 strain (A/Wisconsin/67/2005). Immunological parameters and local reactions and adverse events were monitored.

This study shows important differences in protective immune responses between these vaccines. WIV vaccines elicit the highest antibody levels and a mixed,  $T_H1/T_H2$ , response which indicates cellular immune system activation. Cellular immune responses are thought to be pivotal for protection against heterologous influenza viruses. Trivalent WIV vaccine proved to be safe for use in ferrets. These results affirm the potential of cell culture-grown whole influenza virus vaccines.

4-012

#### Induction of cross-clade long term immunity in mice by different prime and prime & boost immunization schedules with H5N1 whole virus candidate vaccines

**Howard, M.K.;** Nicolas, Sabarth; van Maurik, A.; Savidis-Dacho, H.; Grillberger, L.; Tauer, C.; Reiter, M.; Mundt, W.; Barrett, P.N.; Kistner, O.

Baxter Innovations GmbH, Austria

Since 2003, 371 cases of human infections with the H5N1 influenza virus have been reported, raising the threat of a new pandemic. Baxter has produced H5N1 whole virus candidate vaccines against clade 1 (A/Vietnam/1203/2004) and clade 2 (A/Indonesia/05/2005) strains at an industrial scale using its serum protein-free Vero cell fermenter technology. These candidate vaccines have been shown to be highly immunogenic in small animal models and human clinical studies and are cross-protective in mice and ferrets. Moreover, heterologous prime-boost immunisation schemes using the A/Vietnam/1203/2004 and A/Indonesia/05/2005 vaccine resulted in broad anti-H5N1 immune responses with excellent cross-neutralization activities and cross-protectivity against representative H5N1 viruses of clade 1, clade 2, and subclades 1 and 2.

In the event of a pandemic, huge quantities of vaccine have to be available, strategies to save the required doses have to be in place, and there must be usage of highly efficacious vaccines. In order to investigate dose-saving strategies, we varied the immunisation schedule applying a single immunisation only. Vaccination of CD1 mice with a single dose of the A/Vietnam/1203/2004 or A/Indonesia/05/2005 vaccine induced a strong humoral immune response and provided 100% protection three weeks post-immunisation. Moreover, cross-clade reactive antibodies were detectable and strong cross-protection observed.

High-risk groups like medical personnel are considered to be vaccinated against H5N1 virus in the current pre-pandemic phase already, and therefore long-lasting immunity against H5N1 virus is of particular interest which is what we investigated here. Indeed, 2-shot and even 1-shot immunization schemes induced stable cross-reactive antibody titers and conferred protection against homologous A/Vietnam/1203/2004 and heterologous A/Indonesia/05/2005 virus challenges more than 6 months after the last vaccination.

4-013

#### 1918 pandemic influenza H1N1 DNA vaccine induces protection in ferrets against 2007 H1N1 virus infection

**Bragstad, K.<sup>1</sup>;** Martel, C.<sup>2</sup>; Nielsen, L.P.<sup>1</sup>; Aasted, B.<sup>2</sup>; Fomsgaard, A.<sup>1</sup>

<sup>1</sup>Department of Virology, Statens Serum Institut, Denmark; <sup>2</sup>Department of Veterinary Pathobiology, Faculty of Life Sciences, University of Copenhagen, Denmark

Influenza vaccines with the ability to induce immune responses cross-reacting with drifted virus variants would be very advantageous for vaccine development against seasonal and emerging new strains. We demonstrate that gene gun administrated DNA vaccine encoding HA and NA and/or NP and M proteins of the H1N1 pandemic virus from 1918 induce protection in ferrets against infection with a H1N1 (A/New Caledonia/20/99(H1N1)) virus which was included in the conventional vaccine for the 2006-2007 season. The viruses are separated by a time interval of 89 years and differ by 21.2% in the HA1 protein. These results suggest not only the unique ability of the DNA vaccines but perhaps also natural infection to induce cross-protective responses against even extremely drifted virus variants.

4-014

## Priming effect of an H5N1 pre-pandemic vaccine in a mouse model

**Ikeno, D.I.<sup>1</sup>; Kimachi, K.K.<sup>1</sup>; Kudo, Y.K.<sup>1</sup>; Goto, S.G.<sup>1</sup>; Itamura, S.I.<sup>2</sup>; Odagiri, T.O.<sup>2</sup>; Tashiro, M.T.<sup>2</sup>; Kino, Y.K.<sup>1</sup>**

<sup>1</sup>The Chemo-Sero-Therapeutic Research Institute, Japan; <sup>2</sup>National Institute of Infectious Diseases, Japan

**Purpose:** The pandemic influenza preparedness action plan of Japan stipulates that, when the Minister of Health, Labour and Welfare declares phase 4, health care workers and workers in public services could be vaccinated with pre-pandemic vaccines as an urgency measure with the hope that immunological priming to a pandemic vaccine would occur even though such pre-pandemic vaccines are likely to have antigenic differences to the pandemic strain. However, it is not known what immune responses additional vaccination may elicit and how many vaccinations are needed. In this mouse model study, in order to test the priming effect of the pre-pandemic vaccine, the immune response after vaccination of both the pre-pandemic vaccine (priming effect) and the pandemic vaccine (booster effect) was evaluated.

**Method:** Mice were primed twice with a vaccine (NIBRG-14, derived from A/Vietnam/1194/2004 (H5N1), clade1) or with PBS as a control, and boosted with a different vaccine (Indo05/PR8-RG2, derived from A/Indonesia/5/2005 (H5N1), clade2-1) 4 months later. For the priming vaccine, an alum-adsorbed whole virion vaccine was used. For the booster vaccines, 4 different formulations (plain whole, plain split, alum-adsorbed whole or alum-adsorbed split) were used. Serum HI antibody titers to the clade1 and clade2-1 vaccine viruses were measured before and after the booster injections.

**Result and Discussion:** Mice primed twice with the clade1 vaccine followed by a single booster injection with the clade2-1 vaccine produced a strong and broad HI antibody response to both clades of virus. In contrast, the control group required two shots of the clade2-1 booster vaccines to induce an HI response to the clade2-1 virus and the HI titers were lower than those of the primed group. Little cross reactive antibody response was detected in the control group for the clade1 virus. Furthermore, the non-adsorbed split vaccine elicited a much stronger booster reaction than did the alum-adsorbed whole vaccine whereas, the non-primed groups showed the opposite results.

The results support the possible usefulness of priming with a pre-pandemic vaccine that could provide stronger and broader immunity with a single booster injection of a pandemic vaccine. Moreover, the plain split vaccine is probably one of the best formulations for use as a booster pandemic vaccine. Our data would be helpful in considering a vaccine regimen for pandemic preparedness.

Priming	Booster (Indo05/PR8-RG2)	Indo05/PR8-RG2			NIBRG-14		
		Pre	1st	2nd	Pre	1st	2nd
NIBRG-14	Whole+AL	25.9	190.3	264.6	207.5	1395.8	1076.3
	Whole	18.3	134.5	293.4	269.1	1974.0	1810.2
	Split+AL	21.8	349.0	761.1	246.8	3948.1 *	3948.1 **
	Split	16.8	830.0 *	1395.8 **	269.1	3948.1 *	3948.1 **
PBS	Whole+AL	5.0	16.8	174.5	5.0	5.0	5.0
	Whole	5.0	7.7 *	67.2 *	5.0	5.0	5.0
	Split+AL	5.0	6.5 *	146.7 *	5.0	5.0	5.5
	Split	5.0	5.0 **	26.9 **	5.0	5.0	5.0

Priming vaccines were injected twice with a three week interval. After 4 months, booster vaccines were injected twice with a three week interval. Serum samples were obtained before first booster vaccination (Pre), before second booster vaccination (1st), and 2 weeks after the second booster vaccination (2nd). All dosages were 0.2 ugHA/head. The whole plus alum-adsorbed group was compared to each of the other groups using a Dunnett test (\*:p<0.05, \*\*:p<0.01).

### Acknowledgements:

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### MF59TM-Adjuvanted H5N1 Subunit Vaccine induces a high frequency of Th1 Effector / Memory CD4 T-Cells which persist over time

**Borgogni, E.<sup>1</sup>;** Castellino, F.<sup>1</sup>; Galli, G.<sup>1</sup>; Zedda, L.<sup>1</sup>; Nuti, S.<sup>2</sup>; Tavarini, S.<sup>2</sup>; Bardelli, M.<sup>2</sup>; Malzone, C.<sup>2</sup>; Sammiceli, C.<sup>3</sup>; Praus, M.<sup>4</sup>; Hilbert, A.<sup>4</sup>; Montomoli, E.<sup>5</sup>; Giotti, M.<sup>6</sup>; Gentile, C.<sup>5</sup>; Brauer, V.<sup>4</sup>; Banzhoff, A.<sup>4</sup>; Rappuoli, R.<sup>2</sup>; Del Giudice, G.<sup>2</sup>

<sup>1</sup>Novartis Vaccines, Siena, Italy; <sup>2</sup>Novartis Vaccines, Siena, Italy; <sup>3</sup>Novartis Vaccines, Siena, Italy; <sup>4</sup>Novartis Vaccines, Marburg, Germany; <sup>5</sup>Univ. of Siena, Siena, Italy; <sup>6</sup>ASL7, Siena, Italy

**Background:** Vaccines against potentially pandemic influenza viruses must elicit a strong and broad immune response; however, H5N1 vaccines, even those adjuvanted with alum, are poorly immunogenic. The oil in-water adjuvant, MF59, has a large safety database and proven efficacy at increasing protective antibody levels with low antigen doses. This study assessed both the antibody response and the priming of H5 specific CD4+ T-cells after vaccination with MF59 adjuvanted H5N1.

**Methods:** Adults received two doses of MF59 adjuvanted-H5N1 (A/Vietnam/1194/2004) vaccine containing either 7.5 fYg (n=14) or 15 fYg (n=13) of antigen, or 15 fYg (n=13) of non-adjuvanted subunit H5N1. In each group, a booster dose was given 6 months later. Blood samples were taken at days 1, 22, 43, 202, 223 and 382. After a short in vitro pulse with a pool of peptides covering the entire H5 sequence or with the H5N1 antigen preparation, the frequency and functionality of H5-specific CD4+ T-cells was determined by intracellular staining via polychromatic FACS analysis (fluorescence activated cell sorter). Anti-H5 antibodies were titrated by microneutralization and single radial hemolysis.

**Results:** MF59-adjuvanted H5N1 vaccines enhanced both neutralizing antibody and CD4+ T-cell responses to H5N1. Both MF59-adjuvanted H5N1 vaccines primed H5-specific CD4+ T-cells with a Th1 effector/memory phenotype (IL-13- IL-2+ IFN- $\gamma$  + TNF- $\gamma$  +)  $f|f|fnfn$ . The rise in H5-CD4+ T-cells was detected after the first dose whereas the increase in neutralizing antibodies was detectable after two immunizations. H5-CD4+ T-cells remained above preimmune levels at day 223 and were further expanded by the booster dose given six months later.

**Conclusions:** MF59-adjuvanted H5N1 vaccines induced a CD4+ Th1 immune response and primed a population of H5-specific CD4+ T-cells with a Th1 effector/memory memory phenotype. The safety and immunogenicity profile of MF59- H5N1 vaccines make them ideal candidates for pre-pandemic immunization.

### Study of the efficacy of cortisol in increasing the yield of cold-adapted reassortants for seasonal and pandemic live attenuated influenza vaccine production

**Rekstin, Andrey;** Rudenko, L.G.

Institute of Experimental Medicine RAMS, Russian Federation

The WHO has stated that there is insufficient capacity to produce and supply vaccines to meet an influenza pandemic on a global scale.

It is known that the addition of cortisol/glycocorticosteroids subsequent to inoculation of eggs with A and B influenza viruses may increase the viability and infectious titers. The objective of this study was to improve the magnitude of infectious titres reached in embryonated chicken eggs inoculated with cold-adapted live attenuated influenza vaccine (LAIV) strains based on the A/Leningrad/134/17/57 (H2N2) master strain. In the first set of experiments, influenza A wild-type parental virus A/duck/Potsdam/1402-6/86 (H5N2) and cold-adapted reassortant A/17/duck/Potsdam/86/92 (H5N2) were inoculated into the allantoic cavity of eggs. The dose was  $10^3$  EID<sub>50</sub> for each virus. After 24 hour incubation, cortisol suspensions (0.01 -1 mg/egg) were inoculated in both groups. After a further 24 hours' incubation, influenza A viruses were harvested. Infectious titers (virus yields) obtained in the cortisol-treated eggs and untreated control eggs infected with influenza H5N2 viruses were determined. It was found that addition of cortisol did not affect the yield of wild-type parental virus A/duck/Potsdam/1402-6/86 (H5N2) virus; but that there was a dose-dependent effect of the cortisol on the yield of cold-adapted reassortant A/17/duck/Potsdam/86/92 (H5N2) virus. Addition of 1 mg of cortisol increased the yield of H5N2 cold-adapted reassortant from 9.5 lgEID<sub>50</sub>/ml to 11.5 lgEID<sub>50</sub>/ml.

However, the addition of cortisol after initial virus inoculation is not a practical approach for a vaccine manufacturing process, as it is an additional step in the vaccine production process. It was suggested that cortisol be added at the same time as inoculation with cold-adapted reassortant influenza strain, and that the effect on virus yield be studied. Infectivity of influenza A and B viruses depends on a number of genetic and host factors and might vary from strain to strain. We therefore tested cortisol's effect on virus yield with a number of seasonal cold-adapted reassortant H1N1 and H3N2 influenza viruses. We used several doses of cortisol ranging from 0.005 - 1 mg/egg to investigate whether the effect of cortisol on virus yield is dose-dependent and to assess which cortisol dose produced optimum virus growth.

This study indicates that cortisol can have a significant effect on infectious titers of cold-adapted vaccine reassortant strains. There are some variations dependent on the strain used, concentration of cortisol and egg quality. The data suggests that the addition of cortisol can increase the yield of cold-adapted reassortant viruses from 0.5 to 2 lgEID<sub>50</sub>/ml.

4-017

# **Development of a live influenza polyvalent vaccine containing cold-adapted A (H5N2) vaccine candidate and seasonal reassortant influenza strains (experimental model)**

**Desheva, Julia**; Smolonogina, T.A.; Sergeeva, M.V.; Rudenko, L.G.

*Institute of Experimental Medicine, RAMS, Russian Federation*

Use of polyvalent live-attenuated influenza vaccines (LAIV) may be complicated by interference between vaccine strains. It has been shown previously that interference can be overcome using equal concentrations of vaccine viruses (Romanova, *et al.*, 1993). A clinical study of a trivalent LAIV given to pre-school children has shown an absence of interference between all three seasonal vaccine components (Desheva, *et al.*, 1999). The aim of this study was to evaluate an influenza pandemic A(H5N2) cold-adapted vaccine candidate in experimental co-infection with seasonal cold-adapted (ca) vaccine strains (H1N1, H3N2 and B). The A/17/Duck/Potsdam/86/92(H5N2) ca reassortant virus (Len17/H5) contained the HA gene from non-pathogenic A/Duck/Potsdam/1402-6/86(H5N2) avian virus. The other genes were from the ca H2N2 master-strain (MS) A/Leningrad/134/17/57 (Len/17).

Developing chicken embryos were inoculated with Len17/H5 and Len/17-based H1N1 reassortant, separately or in combination, at infectious doses of 3.5-7.5 lg EID<sub>50</sub>/ml for each virus. The yield was titrated using polyclonal rat H1 and H5 antiserum with 1:40 working titer or normal rat serum. It was found that the Len17/H5 and ca H1N1 reassortant viruses did not interfere with each other in the mixed infection of chicken embryos.

A second study was the reproduction of Len17/H5 in the lungs of CBA mice after inoculation separately with ca H1N1 reassortant or all three seasonal ca vaccine strains (H1N1, H3N2 and B) in combination. The viruses were inoculated intranasally at an infectious dose of 10<sup>6</sup> EID<sub>50</sub>. Lungs were collected on day 3 p.i. from euthanized mice. Sera were collected 28 days p.i. There was no increase of body weight loss (3-3.5%) or increase in virus mix isolation from mouse lungs when Len17/H5 was added to three seasonal reassortant viruses. Using serum H5 virus-specific IgG levels (lg) as a measure, no interference between H5N2 and H1N1 Len/17-based reassortants was found. However after Len17/H5 was inoculated as a tetra-vaccine, serum IgG to H5 were 10-fold lower compared to separate inoculation.

Conclusion: Len/17-based H1N1 and H5N2 ca reassortants inoculated in equal doses did not interfere with each other in chicken embryos. Len17/H5 was safe when given to mice in a tetra-vaccine but there was lower immunogenicity compared to a mono-vaccine.

4-018

# **Screening of reassortant influenza viruses to be used in Live Attenuated Influenza Vaccine (LAIV)**

**Voeten, J.T.M.**<sup>1</sup>; Kiseleva, I.V.<sup>2</sup>; Teley, L.C.P.<sup>1</sup>; Heldens, J.G.M.<sup>1</sup>; van den Bosch, J.F.<sup>1</sup>

<sup>1</sup>Nobilon Schering-Plough, Netherlands; <sup>2</sup>Institute of Experimental Medicine, RAMS, Russian Federation

The cold-adapted (ca), temperature-sensitive (ts) and attenuated (att) influenza viruses A/Leningrad/134/17/57 (H2N2) and B/USSR/60/69 are being used as Master Donor Viruses (MDVs) in the generation of influenza A and B vaccine viruses to be used in Live Attenuated Influenza Vaccine (LAIV). Vaccine viruses are reassortant viruses obtained after co-infection of the MDVs and current wild type (wt) influenza A and B viruses that display the surface antigens of the wt virus and that inherited the genes from the MDV conferring the ca/ts/att phenotype. As time to produce vaccines is limited and reassortant events following co-infection may yield numerous combinations of MDV and wt virus genes, it is important to have tools available allowing rapid screening of viruses for their genetic constellation. RNA electrophoresis and RFLP analysis have been used to screen reassortant viruses but these techniques are laborious and results may not be unambiguous and hence be difficult to interpret. RT-PCR using virus (sub) type and gene segment specific primers has been shown to be a suitable and easy method to demonstrate from which virus (MDV or wt) a gene segment originates. After RNA isolation, cDNA is made of all gene segments in a single reaction using universal primers which is subsequently used in all PCR reactions. Screening typically starts with the surface antigens HA and NA. Only viruses containing HA and NA of the wt virus, will be screened for the presence of MDV genes, beginning with the polymerase genes. In this way initial screening of numerous reassortant viruses is narrowed down rapidly to find the proper reassortants to be included in LAIV.

### Gene expression profiling of dendritic cells exposed to influenza virus or different influenza vaccine candidates

**Pool, Judith;** de Vries-Idema, J.; Wilschut, J.; Huckriede, A.

UMCG, Netherlands

Understanding of the biological mechanisms involved in the reaction of cells of the immune system to influenza virus or vaccines is needed to provide a rational basis for further improvement of current vaccines. In order to assist this need we compared gene expression profiles induced by active virus or current influenza vaccine formulations in vitro. Dendritic cells (DCs) are sentinels of the innate immune system on the one hand and the most important antigen presenting cells of the adaptive immune system on the other hand. They are thus intimately involved in the pathogenesis of influenza as well as in the response to influenza vaccines. We therefore set out to evaluate changes in gene expression induced in these cells by infection with active virus (AV) or by exposure to whole inactivated virus (WIV), split virus (SV) or subunit (SU) vaccine. Myeloid dendritic cells (mDCs) were generated from murine bone marrow by culture in the presence of a recombinant granulocyte-macrophage colony stimulating factor (GM-CSF). Vaccines derived from two influenza strains were included in the study to enable the evaluation of strain-specific differences in the modulation of gene expression. Included strains comprised an H3N2 strain (A/Panama/2007/99) and the H5N1 vaccine strain NIBRG-14. Cells were exposed (in duplicate) to AV, WIV, SV, or SU vaccine (all standardized on the amount of HA) or buffer (PBS) and followed in time. At 4, 12 and 24 hours post-exposure, cells were harvested for FACS analysis and gene expression analysis. Cell supernatants were collected for multiplex cytokine determination. FACS analysis of various markers confirmed the identity of mDC and indicated the maturation of the cells in response to virus or vaccine formulations. In general, AV and WIV induced stronger upregulation of activation markers than SU vaccine. Similarly, DCs exposed to AV or WIV produced more cytokines than cells exposed to SU. Analysis of gene expression profiles using Affymetrix microarrays revealed large numbers of differentially expressed genes ( $p < 0.05$ ) under all conditions ( $> 1000$ ) except SU that revealed lower number of genes ( $> 500$  genes). In most situations, the number of genes that were downregulated after treatment was higher than the number of upregulated genes. The number of differentially regulated genes increased until  $t = 12$ h and remained stable or decreased slightly in the next 12 hours. Interestingly, AV and WIV induced similar changes in gene expression. Most of the genes differentially regulated were shared between both conditions. In contrast, there was only limited overlap in differentially regulated genes between AV and SU or WIV and SU, respectively. Our preliminary analyses indicate that virus replication as is possible in AV-infected DCs has less effect

on gene expression than the exposure of the cells to all viral components as achieved with AV as well as WIV. Further analysis continues to specify the differences and similarities between the different vaccine formulations and active virus. Moreover, the effect of the vaccine strain on the observed modifications in gene regulation will be evaluated. Given the large differences in immunogenicity (high for A/Pan, low for NIBRG-14) these data might reveal which signaling pathways should be triggered to elicit strong immune responses. With these results and our future analyses, we hope to create a better understanding of molecular pathways involved in the response to vaccination which can help in the development of more effective vaccines for influenza.

4-022

### Universal cloning system independent of restriction sites and DNA ligation speeds up generation of recombinant influenza A viruses by reverse genetics

**Stech, Jürgen<sup>1</sup>;** Stech, O.<sup>1</sup>; Herwig, A.<sup>2</sup>; Altmeyen, H.<sup>2</sup>; Hundt, J.<sup>1</sup>; Gohrbandt, S.<sup>1</sup>; Klenk, H.D.<sup>2</sup>; Mettenleiter, T.C.<sup>1</sup>

<sup>1</sup>Friedrich-Loeffler-Institut, Germany; <sup>2</sup>Philipps-Universität Marburg, Germany

Reverse genetics has become pivotal in influenza virus research. Basic research studies and vaccine development rely on the rapid generation of tailored recombinant influenza viruses. They are rescued from transfected plasmids encoding the eight influenza virus segments which have been cloned using restriction endonucleases and DNA ligation. However, in some cases suitable restriction cleavage sites are not available. Therefore, we established a cloning method which is universal for any influenza A virus strain and independent of sequence information. This approach is based on an inverse PCR protocol in which the two strands of an amplicon from an influenza A gene segment serve as megaprimers. The prospective insert must contain termini homologous to the regions of the plasmid adjacent to the insertion site. In order to improve the efficiency, we modified the cloning vector by introducing the negative selection marker *ccdB* flanked by the highly conserved influenza A virus gene termini. From five influenza A virus strains (A/Thailand/1(KAN-1)/2004 (H5N1), A/Swine/Belzig/2/2001 (H1N1), A/Duck/Ukraine/1/1963 (H3N8), A/HongKong/1/1968 (H3N2), and A/Chicken/Emirates/R66/2002 (H9N2)), we cloned all eight genomic segments independent of sequence information amounting to 40 successfully cloned influenza genes. This approach allows fast cloning of all segments from any influenza A strain without knowledge of the genome sequence. If the PCR amplicon ends are homologous to plasmid annealing sites only, this approach is suitable for uniform and efficient cloning of any insert with conserved termini.

4-023

# **A new approach to an attenuated live influenza vaccine: Caspase-dependent intracellular cleavage of influenza viral proteins**

Lee, Kwang-Hee; **Jang, Young Ho**; Seong, B.L.

Department of Biotechnology, College of Engineering, Yonsei University, Republic of Korea

Influenza virus induces apoptosis in infected cells, and during apoptosis activated caspases cleave their substrate proteins that have a caspase recognition peptide sequence. A caspase-dependent N-terminal cleavage of human influenza NP protein has also been reported.

In this study we introduced an artificial caspase cleavage site into the influenza viral proteins with a view to developing a new attenuating component required for construction of influenza live vaccine strain. During apoptosis of virus-infected cells, activated caspase would recognize and cleave the viral proteins resulting in a decrease of viral titre, infectivity or both, depending on cleaved proteins. This mutant virus can be used as new influenza live vaccine because of its reduced pathogenicity and infectivity.

By using reverse genetics we made four different caspase mutant viruses that carry artificial caspase cleavage sites in NP or NS1 protein. Mutant NP and NS1 proteins were efficiently cleaved by caspase even at early infection time, and these mutant viruses showed decreased plaque forming ability and reduced replication in MDCK cells compared to parental wild type virus. Although caspase mutants are attenuated in MDCK cells, their growth in embryonated eggs was as robust as wild type virus. The mutant viruses are being evaluated for safety, immunogenicity, and protective efficacy in a mouse model. This attenuating component could be further combined with pre-existing attenuating devices, for example, cold adaptation, for more safe live attenuated influenza vaccine.

4-024

# **Intramuscular immunization with low dose adjuvanted detergent-split H5N1 influenza vaccine protects ferrets against intratracheal challenge with wild-type homologous virus**

**Mallett, C.<sup>1</sup>**; Baras, B.<sup>2</sup>; Mossman, S.<sup>2</sup>; Stittelaar, K.<sup>3</sup>; Simon, J.<sup>3</sup>; Osterhaus, A.<sup>3</sup>; Burt, D.<sup>1</sup>; Fries, L.<sup>4</sup>; Kenney, R.<sup>4</sup>

<sup>1</sup>GlaxoSmithKline Biologicals, Canada; <sup>2</sup>GlaxoSmithKline Biologicals, Belgium; <sup>3</sup>ViroClinics, Netherlands; <sup>4</sup>GlaxoSmithKline Biologicals, USA

**Background and Aim:** Adjuvantation is considered a key antigen-sparing strategy for pre-pandemic and pandemic influenza vaccines. The following study was undertaken to evaluate preclinically the immunogenicity and efficacy of low doses of an H5N1 vaccine composed of monovalent detergent-split antigen formulated with AS03 adjuvant in a ferret homologous challenge model.

**Study Design:** Seronegative ferrets (six per group) were immunized intramuscularly on days 0 and 21 with 7.5, 3.8, or 1.9 mcg of detergent-split A/Indonesia/5/05 antigen (dosing based on HA) formulated with the AS03 proprietary oil-in-water emulsion-based Adjuvant System. Control groups received 7.5 mcg A/Indonesia split antigen alone or AS03 adjuvant alone. All animals were bled on days 0, 21 and 42 to determine the pre-challenge serum neutralization titers to the immunizing strain as well as three H5N1 drift variants. On day 49, all animals were challenged intratracheally with one hundred thousand TCID<sub>50</sub> of wild-type A/Indonesia/5/05 virus (clade 2, subclade 1) under BSL-3 containment. All animals were monitored for morbidity, mortality and viral shedding in the upper respiratory tract during a 5-day post-challenge observation period. On day 54, all surviving animals were euthanized and viral load and histopathological changes in lung tissue were determined.

**Results:** At all three antigen doses formulated with AS03 adjuvant, the antibody titers to the immunizing strain were clearly boosted after the second immunization. However, there was no clear antigen dose response seen following the second immunization. In addition, the three antigen doses formulated with AS03 adjuvant were each able to elicit cross-neutralizing antibodies following the second immunization to A/Vietnam/1194/04 (clade 1), A/turkey/Turkey/1/05 (clade 2, subclade 2), and A/Anhui/1/05 (clade 2, subclade 3). By comparison with the active groups, there was no enhancement of the antibody response to the immunizing strain following the second immunization in the control group that received antigen alone, nor were cross-neutralizing antibody responses elicited in this group.

There was 100% survival in all three active groups which included the animals that received 1.9 mcg antigen dose, whereas antigen alone and AS03 adjuvant alone control groups exhibited 50% survival (50% moribund euthanasia) and 17% survival (50% moribund euthanasia and 33% mortality), respectively.

Virus recovery from lung tissue, determined by titration on MDCK cells, was negative for all three active groups. In antigen alone and the ASO<sub>3</sub> adjuvant alone control groups, virus was recovered from lung tissue in 67% and 100% of the animals, respectively. By comparison with the lung, viral shedding in the pharynx was also negative for all three active groups. In antigen alone and ASO<sub>3</sub> adjuvant alone control groups, virus was recovered from the pharynx in 33% and 83% of the animals, respectively.

**Conclusion:** In a ferret H5N1 homologous challenge model, low doses of detergent-split antigen (as low as 1.9 mcg of HA) formulated with ASO<sub>3</sub> adjuvant elicited neutralizing antibodies to homologous and heterologous H5N1 viruses, which supports its use in a pre-pandemic immunization strategy. This adjuvanted vaccine induced significantly higher protection, as assessed by increased survival and reduced viral burden in the lower and upper respiratory tract, suggesting a potential role in the reduction of virus transmission. Additional studies are being performed with low doses of this vaccine in a H5N1 heterologous challenge model.

4-025

#### Study of the immunogenicity of experimental avian influenza subunit vaccine for mucosal immunization

**Berezin, V.E.;** Bogoyavlenskiy, A.P.; Tolmacheva, V.P.; Khudiakova, S.S.; Zaitceva, I.A.; Tustikbaeva, G.B.; Omirtaeva, E.S.; Alexyuk, P.G.; Korotetskiy, I.S.

*Institute of Microbiology and Virology, Kazakhstan*

There is an urgent need to develop an influenza vaccine for needle-free mucosal immunization for preparedness for an impending flu pandemic. This vaccine should stimulate an effective systemic and local mucosal immunity and could be administered intranasally as an alternative for subcutaneous immunization. As was shown earlier, immunostimulating complexes assembled virus antigens and the adjuvant active saponin Quil A, isolated from the bark of the South American tree *Quillaja saponaria* Molina, could initiate a wide range of antigen-specific immune responses including humoral and CD4/CD8 cell-mediated responses, and elicitation of mucosal immune response through various routes of vaccination including intranasal immunization (Morein and Abasugra, 2004). Our preliminary study also demonstrated that immunostimulating complexes incorporated influenza virus hemagglutinin and neuraminidase antigens and purified saponins isolated from plants indigenous to Kazakhstan could stimulate high levels of antibody immune responses, initiate IL2 and IFN-gamma production and provide protection against influenza virus infection after intranasal immunization (Berezin *et al.*, 2007, 2008). In the research presented, avian influenza virus subunit vaccine

based immunostimulating complexes incorporated purified hemagglutinin and neuraminidase antigens and plant adjuvants GG-6 and AH-6 isolated from *Glycyrrhiza glabra* and *Aesculus hippocastanum*, plants indigenous to Kazakhstan, have been studied as an experimental vaccine for mucosal immunization on a chicken model. Low toxicity immunostimulating saponins GG-6 and AH-6 have been isolated from plant extracts and purified by HPLC fractionation. Avian influenza virus strain A/FPV/Rostok/34 (H7N1) was grown in 9-day old chicken embryos and purified by centrifugation in sucrose gradient 20-60%. External HA and NA antigens were isolated from purified virus by treatment of non-ionic MESK detergent. Immunostimulating complexes incorporated isolated HA+NA antigens, lipids and purified GG-6/AH-6 saponins or saponin Quil A (Isconova AB, Sweden) which were assembled using a detergent dialysis technique. Two-week old chickens were immunized intranasally with a dose of 3 ug of HA+NA antigens per bird. Two weeks after single immunization, IgG and IgM antibodies were examined in the chicken's sera and birds were challenged with 100 EID<sub>50</sub> of A/FPV/Rostock/34 influenza virus. The results of the study have shown that the avian influenza virus subunit vaccine containing immunostimulating complexes assembled hemagglutinin and neuraminidase antigens and purified plant adjuvants could induce high levels of IgG and IgM antibody and protect birds against the challenge of the highly pathogenic H7N1 avian influenza virus after single intranasal immunization. Complexes incorporated saponin Quil A and prevented infection in 75% of chickens, vaccine possessed saponin AH-6 and protected 90% of chickens and vaccine contained saponin GG-6 and protected 85% of chickens. In the same immunization experiments, intranasal inoculation of a pure HA+NA subunit vaccine or inactivated whole virus vaccine without adjuvants protected only 20-25% of chickens whereas immunization of the HA+NA vaccine mixed with alum hydroxide adjuvant protected 40% of chickens. The results obtained have shown good potential for the immunostimulating complexes delivery system for the development of a highly immunogenic and safe influenza subunit vaccine for mucosal immunization. The research was supported by the ARS USDA-ISTC partner project #K-747p.

4-026



# **Generation of modified influenza A/Vietnam/1203/04 viruses containing truncated NS1 proteins as live attenuated H5N1 vaccine candidates**

**Steel, John**; Lowen, A.C.; Albrecht, R.; García-Sastre, A.; Palese, P.

*Mount Sinai School of Medicine, USA*

Outbreaks of avian influenza in birds in Asia, Africa, the Middle East and Europe continue to pose a threat to poultry in these regions. Towards the development of an effective vaccine against HPAI for use in poultry, a set of experimental live attenuated vaccine viruses based on recombinant influenza A/Vietnam/1203/04 (H5N1) was generated through reverse genetics techniques. All viruses were engineered to express an HA protein in which the polybasic cleavage site had been removed. Viruses which possessed a full-length NS1 or a C-terminally truncated NS1 protein of 73, 99 or 126 amino acids were generated. Viruses with each NS genotype were combined with a PB2 polymerase gene which carried either a lysine or a glutamic acid at position 627. We predicted that glutamic acid at position 627 of PB2 would attenuate the virus in mammalian hosts, thus increasing the safety of the vaccine. Each virus grew to high titers in embryonated chicken eggs but was attenuated in mammalian cell culture. Induction of elevated levels of interferon beta by all viruses possessing truncations in the NS1 protein was demonstrated by interferon bioassay. All recombinant viruses were furthermore found to be highly attenuated in a mouse model. Vaccination of mice with a single dose of  $10^6$  EID<sub>50</sub> of any virus conferred complete protection from death upon challenge with a mouse lethal virus expressing H5N1 HA and NA protein. Additionally, those viruses that were more replication competent in mammalian cell culture also protected challenged mice against signs of disease in a dose dependent fashion. Thus, recombinant influenza A/Vietnam/1203/04 (H5N1) viruses possessing truncations in the NS1 protein display characteristics desirable for a live attenuated vaccine and may hold potential as vaccine candidates in poultry as well as in mammalian hosts.

4-028

### Plant-produced HA from A/Indonesia/05/05 protects ferrets against homologous challenge infection

**Yusibov, Vidadi<sup>1</sup>**; Shoji, Y.<sup>2</sup>; Farrance, C.E.<sup>2</sup>; Bi, H.<sup>2</sup>; Shamloul, M.<sup>2</sup>; Green, B.<sup>2</sup>; Manceva, S.<sup>2</sup>; Rhee, A.<sup>2</sup>; Ugulava, N.<sup>2</sup>; Roy, G.<sup>2</sup>; Rabindran, S.<sup>2</sup>; Musychuk, K.<sup>2</sup>; Chichester, J.A.<sup>2</sup>; Mett, V.<sup>2</sup>

<sup>1</sup>Fraunhofer USA Center for Molecular Biotechnology, USA; <sup>2</sup>Fraunhofer USA CMB, USA

Highly pathogenic avian influenza viruses of the H5N1 subtype have been identified as a potential pandemic threat by the World Health Organization (WHO). Since the index human case in Guangdong Province China in 1997, these viruses have continued to spread throughout the world, infecting 373 humans to date, with 236 fatalities. Vaccination is the preferred strategy for the prevention and control of influenza infections and several approaches have been used to generate vaccines against clade 1 H5N1 viruses A/Vietnam/1194/2004 and A/Vietnam/1203/2004. In 2006, however, the WHO changed the recommended vaccine strains of H5N1 from clade 1 to clade 2 viruses based on the continued emergence of new strains. Since it is unknown which strain may potentially cause an influenza pandemic, the availability of a system for the rapid engineering and production of vaccines is needed to combat an influenza pandemic. Recently, plants have been used to produce recombinant proteins including vaccines and antibodies. The main advantages of using plant systems for the production of vaccine against HPAI are their independence from pathogenic viruses and egg-based production systems as well as their cost and time efficiency. Here we describe the immunogenicity and protective efficacy of recombinant HA from A/Indonesia/5/2005 produced in *Nicotiana benthamiana* plants. This plant-produced HA induced serum hemagglutination inhibition and virus neutralizing antibody titers in mice. Furthermore, immunization of ferrets with this plant-produced HA provided protection against a homologous virus challenge. These results suggest the utility of our plant-expression system for recombinant influenza vaccine production.

4-029

### Facing the pandemic threat: preclinical development of innovative cross-protective H5N1 influenza virus-like particles

**Landry, Nathalie**; Trepanier, S.; Guay, J.M.; D'Aoust, M.A.; Dargis, M.; Vezina, L.P.

Medicago Inc., Canada

Medicago adapted its proprietary transient expression technology to the production of influenza antigens in plant biomass. It is the first demonstration that the formation of influenza virus-like particle (VLP) structures can be obtained in plants from the sole expression of the hemagglutinin antigens. The transient technology could deliver a vaccine for testing in about a month after the identification and reception of genetic sequences from the pandemic strain. Mice studies with H5N1 influenza VLPs showed that the plant-made vaccine induced cross-protection against lethal challenge with highly pathogenic H5N1 influenza strains of different clades and subclades. Results from studies with ferrets will also be described, as well as product characterization and manufacturing processes. These data will demonstrate the potential of our technology for fast response, dose-sparing and critical surge capacity in an efficient and cost-effective manner in the event of a pandemic outbreak.

4-030



### An influenza A vaccine based on tetrameric ectodomain of matrix protein 2

**Schotsaert, M.A.K.<sup>1</sup>**; De Filette, M.<sup>1</sup>; Martens, W.<sup>1</sup>; Roose, K.<sup>1</sup>; Deroo, T.<sup>1</sup>; Vervalle, F.<sup>1</sup>; Bentahir, M.<sup>1</sup>; Vandekerckhove, J.<sup>2</sup>; Fiers, W.<sup>1</sup>; Saelens, X.<sup>1</sup>

<sup>1</sup>DMBR/VIB/UGent, Belgium; <sup>2</sup>VIB/UGent, Belgium

Matrix protein 2 (M2) of influenza A is a tetrameric type III membrane protein that functions as a proton-selective channel. The extracellular domain (M2e) has remained nearly invariable since the first human influenza strain was isolated in 1933. By linking a modified form of the leucine zipper of the yeast transcription factor GCN4 to M2e, we obtained a recombinant tetrameric protein, M2e-tGCN4. This protein mimics the quaternary structure of the ectodomain of the natural M2 protein. M2e-tGCN4 was purified, biochemically characterized, and used to immunize Balb/c mice. High M2e-specific serum IgG antibody titres were obtained following either intraperitoneal or intranasal administration. Immunized mice were fully protected against a potentially lethal influenza A virus challenge. Antibodies raised by M2e-tGCN4 immunization specifically bound to the surface of influenza-infected cells and to an M2-expressing cell-line. Using an M2e-peptide competition ELISA with M2-expressing cells as the target, we obtained evidence that M2e-tGCN4 induces antibodies that are specific for the native tetrameric M2 ectodomain. Therefore, fusion of an oligomerization domain to the extracellular part of a transmembrane protein allows it to mimic the natural quaternary structure and can promote the induction of oligomer-specific antibodies.

4-031

### Cross-reactive immunity to diverse influenza H5N1 viruses by boosting primed subjects with an antigenically distinct vaccine

**Stephenson, Iain<sup>1</sup>**; Hancock, K.<sup>2</sup>; Hoschler, K.<sup>3</sup>; Praus, M.<sup>4</sup>; Banzhoff, A.<sup>4</sup>; Montomoli, E.<sup>5</sup>; Zambon, M.<sup>3</sup>; Katz, J.<sup>2</sup>; Nicholson, K.<sup>6</sup>

<sup>1</sup>University Hospitals Leicester, UK; <sup>2</sup>CDC, USA; <sup>3</sup>Health Protection Agency, UK; <sup>4</sup>Novartis Vaccines, Germany; <sup>5</sup>University of Siena, Italy; <sup>6</sup>University of Leicester, UK

**Background:** Priming in advance of the next influenza pandemic could reduce vaccine shortages during the first months. Immediate boosting as soon as a pandemic is declared may allow rapid induction of antibody responses satisfying a key component of pandemic preparedness plans.

**Methods:** In an open-label study, 54 subjects received two doses of 7.5µl MF59-adjuvanted influenza A/Vietnam/1194/2004 clade 1 H5N1 vaccine. Twenty-four of the subjects had been primed at least 6 years ago with either MF59-adjuvanted or non-adjuvanted A/duck/Singapore/1997 clade o-like H5N3 vaccine. Pre- and post-antibody responses to antigenically diverse avian H5 viruses were measured by hemagglutination-inhibition (HAI), neutralizing antibody (MN) and single radial hemolysis (SRH).

**Results:** Geometric mean antibody titers and frequency of responses were significantly higher in primed subjects than in unprimed subjects. By day 7 after one dose of vaccine, 80% of MF59-H5 primed recipients achieved seroprotective HAI titers of at least 1:40 to all clade 1, 2.1, 2.2, and 2.3 avian H5 virus variants tested as well as the original A/duck/Singapore/97 clade o-like antigen. Among study participants, responses were greatest at day 14 in those primed with MF59-H5 vaccine with geometric mean antibody titers of 1:378, 1:1754 and 73mm2 to the clade 1 A/Vietnam/2004 vaccine strain and 1:347, 1:2128 and 72mm2 to a clade 2 A/Turkey/2005 variant by HAI, MN and SRH respectively. Serology results at six months are pending.

**Conclusion:** Subjects primed with MF59-adjuvanted vaccine responded significantly better than those primed with conventional vaccine. Among primed subjects, protective cross-reacting antibody titers to diverse H5N1 virus variants were seen by day 7 after a single vaccine dose. These results may help guide policy-makers on the rationale for advance priming and use of pre-pandemic stockpiled vaccines.

## 5 DISEASE SURVEILLANCE & SOCIO ECONOMICS

5-001

### Are children the main transmitters of influenza-like illness in the community? An analysis of data from European sentinel networks

*Elliot, A.J.; Fleming, D.M.*

*Royal College of General Practitioners, UK*

**Introduction:** The administration of influenza vaccine in young children has become routine in some countries. Protecting this age group is the main reason for this policy. However, it has been suggested that controlling the spread of infection in younger age groups limits the transmission of the virus, thus reducing the morbidity, mortality and economic burden associated with influenza infections in older people.

**Aims:** We have previously analysed 40 years of influenza-like illness (ILI) clinical incidence data (winters 1967/68 to 2006/07) collected from the Royal College of General Practitioners Weekly Returns Service (WRS) sentinel surveillance network. In this presentation we further our analysis to data collected from other European sentinel surveillance schemes involved with the European Influenza Surveillance Scheme (EISS).

**Methods:** New episode incidence rates for ILI per 100,000 population were collected from the WRS and other European countries involved with EISS. Data were further analysed by age group (0-4, 5-14, 15-44, 45-64 and 65+). Weeks containing the peak incidence rate of ILI were compared across all age bands and cross correlations tabulated to calculate lag periods between the different series.

**Results:** We have, to date, analysed data from the WRS. The timing of annual influenza epidemics ranged in peak incidence from week 45 (early November, 1993/94) to week 15 (mid-April, 1983/84). The peak clinical incidence rate of ILI was spread across all age groups although the precise burden differed from winter to winter. Lag periods were calculated for ILI and acute bronchitis across all age groups for individual winters, and for the combined 39-year study period. There were no consistently discernable lag periods detected between any age groups for ILI. We will present similar results from other European sentinel networks.

**Discussion:** Provisional analysis has found no evidence of delay between the clinical incidence of ILI in young children and the elderly. Therefore, these current data do not support the routine vaccination of young children as a means of reducing the morbidity, mortality and economic burden associated with influenza infections in older age groups. We therefore conclude that where national policy advocates the vaccination of healthy children, vaccine should be administered purely for the benefit and cost-saving in these young age groups.

5-002

### SENTINEL and non-SENTINEL data on influenza and influenza-like illnesses in Poland in the epidemic season 2007/08

*Romanowska, Magdalena<sup>1</sup>; Nowak, I.<sup>1</sup>; Brydak, L.B.<sup>2</sup>*

*<sup>1</sup>National Influenza Center, National Institute of Public Health - National Institute of Hygiene, Warsaw, Poland; <sup>2</sup>National Influenza Center, National Institute of Public Health - National Institute of Hygiene; Medical University of Warsaw, Poland*

The National Influenza Center (NIC) in Poland is a member of the WHO Global Influenza Surveillance Network (GISN) and European Influenza Surveillance Scheme (EISS). Therefore, one of the most important tasks of NIC is surveillance of influenza that provides information on influenza activity in a given population and season. These data are practically used and necessary for appropriate selection of vaccine strains, studies on the current and new vaccines and antivirals, development of new diagnostic reagents, introduction of effective measures to limit the spread of influenza virus infections, and consequently to limit the number of influenza illnesses, post-influenza complications and deaths. The aim of the study was to present epidemiological and virological data on influenza and influenza-like illnesses (ILI) caused by RSV, parainfluenza and adenovirus in Poland in the epidemic season 2007/08. Since the epidemic season 2004/05, the SENTINEL influenza surveillance system has existed in Poland and the participants in this system are: sixteen regional sanitary-epidemiological stations, i.e. Voivodship Sanitary-Epidemiological Stations (VSESs), a selected number of family physicians representing sixteen voivodships and the NIC as the coordinator. SENTINEL physicians register on a weekly basis the number of cases of influenza-like illness in the age groups: 0-4, 5-14, 15-64 and  $\geq 65$  years and collect specimens. The other source of specimens is hospitals (non-SENTINEL specimens). Physicians send epidemiological data and specimens to an appropriate VSES, where virological tests are performed. All VSESs isolate influenza virus on the MDCK cell line and most of them also perform direct immunofluorescence tests to detect antigens of influenza A, influenza B as well as other respiratory viruses causing influenza-like illnesses such as RSV, parainfluenza type 1, parainfluenza type 2, parainfluenza type 3 and adenovirus. VSESs prepare weekly reports with epidemiological data and reports with virological data and send them to the NIC. Isolates of influenza strains are also sent by VSESs to the NIC for antigenic analysis, which is performed by hemagglutination inhibition tests with reference sheep antisera and ferret antisera received from WHO Collaborating Centres for Reference and Research on Influenza located in the Centers for Disease Control

and Prevention, Atlanta and in the National Institute for Medical Research, London, respectively. On the basis of information received from VSEs, the NIC prepares weekly reports with data for the entire country and sends these to the GISN and EISS. NIC also sends virological data to the Dept. of Epidemiology, NIPH-NIH to be included in the periodical national reports on influenza and influenza-like illnesses. Data presented in this abstract are from the period between week no. 36/2007 and week no. 12/2008. Epidemiological reports and virological reports were received from all sixteen VSEs. Only at the beginning of the season, i.e. in week no. 36/2007, did one of the VSEs not send the reports as the network of physicians was not ready at this time. The weekly number of reporting physicians ranged from 972 to 1108, covering 5.1% of the total population of Poland. The total incidence of influenza and ILI amounted to 2363.2/100,000, while the weekly incidence ranged from 2.9/100,000 (week no. 36/2007) to 166.1/100,000 (week no. 08/2008). The highest incidence was observed in children aged 0-4 years. The number of specimens collected and tested amounted to 1139, including 955 SENTINEL specimens (83.8%) and 184 non-SENTINEL specimens (16.2%). Among 1139 specimens, respiratory infections (influenza, RS, adenovirus parainfluenza) were confirmed in 104 cases (9.1%). Among 955 SENTINEL specimens, 6.6% were positive for influenza and/or other respiratory viral infections, while among non-SENTINEL specimens 22.3% were positive. Among 104 positive specimens, influenza infections were confirmed in 65.4% of cases, RSV – in 26% of cases, parainfluenza type 1 – in 1% of cases, parainfluenza type 2 – in 1% of cases, parainfluenza type 3 – in 2.9% of cases and adenoviruses – in 3.8% of cases. Among influenza infections, 58.8% were caused by type A, and 41.2% by type B. Until week no. 12/2008, eleven influenza virus strains were isolated and antigenically characterized. Six isolated strains were subtyped as influenza A/H1 similar to A/Solomon Islands/3/2006 (H1N1) and five strains were typed as influenza B similar to B/Florida/4/2006. Almost 20 other isolates are still undergoing procedures aiming to produce high amounts of virus of high enough hemagglutinin titer to be able to perform antigenic analysis. Influenza activity observed in Poland between week no. 36/2007 and week no. 12/2008 of the epidemic season 2007/08 was lower than in the same period of the previous epidemic season 2006/07 (incidence of influenza and ILI amounted to 2494.3/100,000 with weekly incidence ranging from 2.9/100,000 in week no. 36/2006 to 265.8/100,000 in week no. 09/2007). Nevertheless, in both epidemic seasons the most affected group were children aged 0-14 years, and the peak of influenza activity was observed in February. Virological data showed that influenza strains of type A co-circulated with strains of type B, while in the season 2006/07 influenza strains of type A were dominant. A significant percentage of infections were also caused by RSV indicating that surveillance of influenza should also include other viruses that are a reason for influenza-like illnesses. Furthermore, a significantly lower percentage of the positive SENTINEL specimens when compared with non-SENTINEL specimens showed that an improvement of the quality

of the virological surveillance is necessary, especially in the area of collection, storage and transport of specimens for obtaining reliable results in laboratory testing.

5-003

### Intervals, triggers, and actions: A new framework for enhancing pandemic influenza planning and response

**Hoelscher, M.<sup>1</sup>; Hwang, I.<sup>1</sup>; Demma, A.<sup>1</sup>; Thompson, W.<sup>1</sup>; Zhou, H.<sup>1</sup>; Lindstrom, S.<sup>1</sup>; Breese, J.<sup>1</sup>; Cox, N.<sup>1</sup>; Polder, J.<sup>2</sup>; Jernigan, D.<sup>1</sup>**

<sup>1</sup>Influenza Division, National Center for Immunization and Respiratory Diseases, CDC, USA; <sup>2</sup>Division of Global Migration and Quarantine, NCPDCID, CDC, USA

**Background:** Available data from the 1918 pandemic show that certain communities were affected differently than others and that implementation of community-wide social distancing efforts early in the pandemic may have lessened the impact on their communities. Recent policies for use of non-pharmaceutical mitigation measures have outlined specific response actions by communities during a pandemic; however, the triggers for ‘when’ to initiate and cease these and other response actions have not been articulated.

**Methods:** To further characterize when various responses should be started and stopped before or at various points during a pandemic of influenza, CDC engaged with public health and laboratory stakeholders to develop a detailed delineation of likely decision points (termed “pandemic intervals and triggers”) along a pandemic epidemiologic curve. The proposed approach was tested during a 48-hour agency-wide exercise and information gathered during this exercise was used to validate and refine the design.

**Results:** Participants developed a response framework which subdivides WHO Pandemic Phase 6 into smaller intervals to refine pandemic planning. The framework was based upon application of an idealized epidemic curve occurring during WHO Phase 6. Five ‘Intervals’ of time were defined, each ‘triggered’ by key epidemiologic events that are anticipated during spread of a pandemic virus. The idealized epidemic curve was divided into: Initiation, Acceleration, Peak Transmission, Deceleration and Resolution. Proposed laboratory and epidemiologic triggers marking the beginning of each interval were chosen to facilitate planning and timing of responses by U.S. national, state and local governments to: limit the spread and effects of the disease; facilitate communication; and provide support to critical infrastructure, such as first responders and hospitals. For each ‘Interval’, various actions and triggers appropriate for that time in the pandemic were described for maximizing effective use of antivirals, vaccines, surveillance systems, diagnostic testing, and community mitigation measures. Detailed laboratory surge

requirements by interval were also calculated. Exercise testing of the framework revealed the critical need for highly sensitive and specific laboratory confirmatory testing and for flexible, low-resource approaches to monitor the progression of the pandemic. U.S. Government planning guidance has now incorporated the 'Intervals' approach.

**Conclusion:** A new planning framework using 'Intervals, Triggers, and Actions' provides a needed enhancement to prior planning and response methods. This new approach accounts for the asynchronous nature of a progressing pandemic by identifying local triggers for initiating and ceasing interventions at the appropriate time for affected communities during WHO Phase 6.

5-005

### Patterns of influenza virus circulation, age- and strain-specific morbidity of influenza in Portugal

**Gonçalves, P.;** Rebelo-de-Andrade, H.; Coelho, A.; Pechirra, P.; Arraiolos, A.; Santos, L.

*Centro Nacional da Gripe, Instituto Nacional de Saúde Dr. Ricardo Jorge, Portugal*

**Background:** The knowledge of the impact of influenza epidemics in Portugal can contribute to management of the disease and implementation of preventive and/or therapeutic measures in future influenza epidemics. It can also contribute to the improvement of clinical and virological influenza monitoring systems.

**Objectives:** The objectives of this study were to evaluate the patterns of influenza virus circulation in Portugal over the past 8 influenza winter seasons (1999/2000 to 2007/2008) and to correlate this information with the incidence of the influenza-like illness in the Portuguese population, particularly in different age groups.

**Methods:** Clinical and epidemiological data were obtained from Influenza-Like Illness (ILI) cases reported to the Department of Epidemiology (DE) and to the National Influenza Centre (NIC) of the National Institute of Health, through the National Influenza Surveillance Programme. The intensity and duration of the epidemic periods were described based on the weekly incidence rates for ILI, calculated by the DE for the general population and for specific age groups (0-4, 5-14, 15-64 and over 65 years). The excess of population with ILI was found as a function of the baseline for the incidence of ILI, which was calculated from data collected over a period of 12 years and is used every winter season to estimate the impact of seasonal influenza epidemics. Virological characterisation of influenza viruses in circulation was achieved by genetic and antigenic analysis of virus isolates obtained from nasopharyngeal swabs, collected from the cases

reported to the NIC.

**Results:** The epidemic periods observed during the 8 influenza winter seasons analysed varied in terms of timing, duration and intensity. Epidemics associated with the circulation of influenza type A (1999/2000, 2001/2002, 2003/2004, 2004/2005 and 2006/2007) usually occurred during December or January, lasting 8 weeks on average (5-11 weeks). When influenza type B were dominant (2000/2001, 2002/2003, 2007/2008), the epidemic periods, when existent, were shorter, lasting 2 weeks on average (0-3 weeks), and occurred at the end of January through February. The higher values of the incidence rates for ILI also occurred when influenza type A was dominant.

In terms of circulation of influenza viruses, influenza type AH3 and B usually alternated every other year. Influenza type AH1 viruses were sporadically detected, with the exception of the 2000/2001 and 2007/2008 seasons when they co-circulated with influenza type B. Influenza A/Panama-like (AH3) viruses circulated until the 2002/2003 season, when they gave way to the A/Fujian-like (AH3) lineage. These viruses have been drifting away from A/Fujian/411/2002, from being similar to A/California/7/2004 during 2004/2005, to being similar to A/Brisbane/10/2007 during 2006/2007. Since the 2000/2001 winter season, Influenza B/Victoria/7/87-like and B/Yamagata/16/88-like viruses have been alternating in their dominance. The influenza AH1 viruses in circulation were similar to the vaccine strain A/NewCaledonia/20/99, not showing substantial genetic differences between them. However, during the last winter season 2007/2008, there was a clear antigenic distancing from the initial A/Solomon Islands/3/2006-like viruses, to the recently detected A/Brisbane/59/2007-like viruses.

Influenza-related morbidity was higher during influenza seasons associated with the predominant circulation of influenza AH3, with an average excess of 556 cases per 100 000 inhabitants, while during influenza B seasons the average excess observed was 36 cases per 100 000 inhabitants.

The data aggregated by age group indicates that during the winters associated with the circulation of influenza B, there was a clear impact of ILI on the 5-14 years age group. This observation was particularly clear for the 2002/2003 season (associated with B/Victoria-like viruses that accounted for over 95% of viruses detected that season), during which the excess population with ILI, in this age group, was above the average (0.53%, average 0.34%). When influenza type A was dominant, the difference in the impact of ILI between age groups was not as evident. The excess population with ILI reached high values (0.41% to 0.64%, on average) for all age groups, particularly for young children (0-4 years) and the elderly (over 65 years), by comparing with influenza B seasons. The excess population by age group for the 1999/2000 and 2006/2007 seasons remained within the average. However, this was not the case for 2001/2002, 2003/2004 and 2004/2005. For the 2001/2002 season, the percentage of the population with ILI was above the average for all age groups. While during 2003/2004, the 0-4 and 5-14 years age groups were the most affected (0.66%, average 0.41%, and 0.98%,

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average 0.64%, respectively), during the 2004/2005 season the adult population (15-64 years, 0.93%, average 0.60%) and the elderly (over 65 years, 0.97%, average 0.48%) suffered the greatest impact of ILI.

**Discussion:** An interesting aspect of this analysis was the alternating pattern with which influenza viruses circulate in the population, not only alternating between types A/B, but also between lineages of the same type. For the first case, during the 8 winter seasons analysed, there was only one exception observed during 2003/2004 and 2004/2005, associated with the introduction of the A/Fujian lineage. For the latter, the circulation of the B/Yamagata and the B/Victoria lineages over this 8-year period is a good example.

While influenza AH1 viruses circulating in the population seem to evolve at a lesser degree within the same timeframe, influenza types AH3 and B continue to evolve, drifting away from the reference strains. The evolution of circulating strains in relation to the vaccine strains will be further analysed and discussed retrospectively.

When relating the circulation of influenza viruses with the incidence of the ILI by age group, the data collected indicates that morbidity was age- and strain-specific, and two main aspects stand out. First, the clear impact that the influenza type B virus exerts on the incidence of the disease among school-aged children. This was particularly evident for the 2002/2003 season, probably associated with the circulation of the B/Victoria lineage after more than one decade of B/Yamagata dominance. Second, all age groups are greatly affected by the disease when type A viruses are dominant. For this case, two situations were observed. On one hand, the percentages of excess population with ILI recorded for the 2001/2002 winter season were above the average for all age groups. The reasons for this observation were not clear and further studies on the antigenic and genetic properties of the A/Panama-like viruses in circulation would then be of interest. On the other hand, the emergence of the A/Fujian lineage during 2003/2004 appears to be related to an increase in the impact of the ILI in the younger age groups, in which residual immunity to these new variants may not have been sufficient to confer protection. During the following winter, this age tendency was reverted and the adults and the elderly were the most affected, a fact that is compatible with the continuous circulation of these variants.

5-006

### Impact of pandemic flu educational sessions on knowledge and acceptance of the pandemic French plan among GPs

**Cohen, Jean Marie**<sup>1</sup>; Lina, B.<sup>2</sup>; Chidiac, C.<sup>3</sup>; Elsayy, A.<sup>4</sup>; Haas, H.<sup>5</sup>; Japhet, C.<sup>6</sup>; Raffi, F.<sup>7</sup>; Bensoussan, J.L.<sup>8</sup>

<sup>1</sup>Open Rome, France; <sup>2</sup>CNR virus Influenzae Lyon, France; <sup>3</sup>CHU Lyon, France; <sup>4</sup>GP, France; <sup>5</sup>CHU Nice, France; <sup>6</sup>UNPF, France; <sup>7</sup>CHU Nantes, France; <sup>8</sup>MG, France

**Objective:** To evaluate the impact of educational sessions organized during 2007 by French health authorities on GPs' knowledge concerning pandemic plans and their influence on seasonal flu management.

**Methods:** In January 2008, SOFRES conducted a survey by phoning 501 GPs and filling out a questionnaire written by a scientific committee.

**Results:** 437/501 (87%) of GPs heard about these sessions and 256 (51%) participated. Information remembered was: general organisation (35%), hygiene measures (45%), epidemiology (24%) and therapeutic strategy (18%). Global satisfaction about the contents was 88%, 82% had the plan available, 70% said they were involved in pandemic management and 45% said they were ready. Only 38% of GPs evaluated the success rate of this plan as high. The pandemic issue was real for 75%. Facing a suspect H5N1 case, 87% of GPs will adopt hygienic measures, 86% will declare the patient, and 86% will adopt barrier measures, including masks (73%), washing hands (43%), gloves (21%), office disinfection (40%) and infected case isolation (10%). However 33% said they would close their office. Since these educational sessions, flu vaccination of GPs themselves increased by 8% and neuraminidase inhibitor prescriptions for seasonal flu increased by 9%.

**Conclusion:** This educational programme did achieve most of its purposes on pandemic influenza management plan knowledge. The caring process for a pandemic flu needs to be improved.

5-007

### Hospitalizations associated with influenza and respiratory syncytial virus in Spain in the period 1997-2005

**Jiménez-Jorge, S.J.J.;** de Mateo Salvador, S.D.M.; Larrauri Amparo, A.L.

National Centre for Epidemiology, Carlos III Institute of Public Health, (CIBERESP), Spain

**Introduction:** Influenza is a major cause of acute respiratory disease. Excess hospitalization associated with influenza has been used extensively to measure influenza severity. However,

co-circulation of other respiratory viruses during the influenza season, such as the respiratory syncytial virus (RSV), makes it difficult to estimate the influenza-associated morbidity burden accurately. Several models have been used to estimate the excess influenza and RSV, based on determining the excess rate during influenza and RSV circulation periods versus baseline periods with lower or no virus activity. The aim of the current study was to evaluate influenza- and RSV-associated hospitalizations in Spain during the period 1997-2005, comparing two different models to estimate virus active periods: the 70% approach defined by Fleming (1) (Model 1) and the rate difference model defined by Izurieta(2,3) (Model 2).

**Methods:** We studied eight winter seasons (1997-1998 to 2004-2005) from week 40 of one year to week 20 of the following year. Virological data were obtained from two different Spanish surveillance systems: 1) the Spanish Influenza Sentinel Surveillance System (SISSS) which covers approximately 85% of the Spanish population, 16 of the 19 autonomous regions, who notify weekly the number of consultations for influenza-like illness (ILI) in their reference populations. In addition, general practitioners regularly send samples for virological confirmation to the sentinel laboratories in their region (17 laboratories in total); and 2) the Microbiological Information System (SIM) which covers approximately 25% of the Spanish population based on the voluntary weekly reports of confirmed microbiological diagnoses, RSV among them, carried out by 42 laboratories (mainly hospital). Study outcomes were weekly hospitalizations from the Minimum Basic Set of Data (MBSD) maintained by the Ministry of Health according to the International Classification of Diseases-9CM for categorizing hospitalizations. Weekly hospitalizations were summarized by the first-listed discharge code of two different categories: pneumonia and influenza (P&I) (480-487), and circulatory and respiratory disease (C&R) (390-459, 460-519). Thus, P&I hospitalizations were included in C&R hospitalizations. For each winter season, we used as the denominator the population on July 1st of the first year of the winter season (Spanish National Institute of Statistics), assuming a stable population throughout the season. Weekly hospitalization rates were averaged in each winter season (from week 40 of one year to week 20 of the next), for each hospitalization category in the different active periods according to virus activity. In model 1, virus active periods were defined as the weeks surrounding the peak week of the respective virus laboratory reports that encompassed a minimum of 70% of the total number of reports submitted to the SISS and MIS respectively, during the winter season. Periods were classified as influenza active weeks, RSV active weeks, combined virus active weeks and baseline (those weeks outside the virus active period). We calculated the apportioned estimate for influenza and RSV from the combined estimates as per Fleming 2007. In model 2, the influenza and RSV virus-active period was defined as the periods of at least two consecutive weeks in which each week accounted for  $\geq 5\%$  of the season's total number of laboratory-confirmed influenza or RSV cases, respectively. The period with

influenza or RSV predominance was defined as the influenza or RSV virus-active weeks with  $< 5\%$  of the season's total number of positive tests for RSV or influenza, respectively. The baseline period was defined as the period of at least two consecutive weeks within week 40-20 in which each week accounted for  $< 5\%$  of the season's total number of influenza and RSV positive reports. Weekly excess hospitalization rates associated with P&I and C&R during influenza and RSV periods were measured relative to baseline periods for model 1 and model 2. For each hospitalization category, the estimated annual cumulative winter excess rate was the average of the difference between the weekly hospitalization rate in the influenza and RSV period over the average weekly hospitalization rate of the baseline period and multiplied by the number of weeks of respective study periods. The resulting annual cumulative excess hospitalization rates for influenza and RSV for model 1 and model 2 were treated as two paired samples, and sample median differences (with 95% CIs) were calculated by application of the non-parametric method for paired differences (as described in Fleming 2007) to determine the significance of differences between excess in influenza versus RSV active periods.

**Results:** Total hospitalization excess for C&R and P&I varied among winter seasons reflecting the seasonal variation in the magnitude and timing circulation of influenza and RSV. We obtained a similar pattern of period specific C&R and P&I hospitalizations rates with the two models, although model 1 gave higher cumulative excess hospitalization rates. The hospitalization excess estimated was higher in influenza than RSV active periods for both C&R and P&I in both models. However, with model 1, we obtained hospitalization excess differences between influenza and RSV active periods 8 and 2 times higher for C&R and P&I, respectively, than with model 2. For C&R hospitalizations, the estimated difference (95% confidence interval) of total excess between the influenza and RSV active period, with model 1, was 39 (16-53) (rate per 100,000 population).

**Conclusions:** Hospitalization burden due to C&R and P&I was higher in influenza than in RSV active periods, in the period 1997-2005 in Spain. Model 1 gave higher hospitalization excess than model 2, probably related to a longer length of the virus' active periods. Ongoing analysis by age group will give more accurate information about influenza and RSV hospitalization burden.

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5-008

### Spread and biological properties of influenza viruses in Russia in the period 2003-2008

**Ivanova, V.I.;** Kurochkina, Y.E.; Bourtseva, E.I.; Trushakova, S.V.; Oskerko, T.A.; Matyusina, R.O.; Slepishkin, A.N.

*D.I. Ivanovsky Research Institute of Virology RAMS, Moscow, Russian Federation*

Influenza surveillance is carried out by the Influenza Ecology and Epidemiology Center (IEEC). It includes the study of virus spread and strain properties. The data presented shows the epidemic strains (n=650) causing the epidemic upsurge of influenza morbidity among people. The strains were received at the IEEC from 19 regions located from the East to West boundary of Russia from December 2003 to April 2008. The spectrum of viruses circulating in these epidemic seasons included influenza A(H1N1), A(H3N2) and B viruses in different combinations. Influenza A(H1N1) viruses were isolated in 2004-2008. The largest number of isolates (n=101) were detected in 2006-2007, when the variants of the previously current reference strains A/New Caledonia/20/99 and new A/Solomon Islands/3/06 started to circulate together. The strains (n=30) closely related to A/Solomon Islands/3/06 were isolated in 2007-2008. Influenza A(H3N2) viruses isolated annually. The number of isolates varied from 78 to 113. The evolution of viruses occurred in the direction A/Fujian/411/02 → A/Kumamoto/102/02 → A/California/7/04 → A/Wisconsin/67/05 → A/Brisbane/10/07. The A/Brisbane/10/07-like strains have been detected since 2007. Influenza B viruses belonging to different lineages had different activities. B/Jamagata/16/88-lineage influenza strains were widespread in 2004-2005 (n=87), 2006-2007 (n=24), 2007-2008 (n=37). Hemagglutinin (HA) of epidemic strains changed as HA of B/Sichuan/379/99 → B/Shanghai/361/02 → B/Florida/4/06. Influenza B viruses of B/Victoria/2/87 lineage were actively isolated in 2005-2006 (n=56), 2006-2007 (n=36). HA of epidemic strains changed as HA of B/Hong Kong /330/02 → B/Malaysia/2506/04. Therefore antigenic properties of epidemic strains, circulating in Russia, were similar to modern reference strains circulating in other countries, but their spread has its own specificities. The virological data were confirmed by serological results. The increases of antibody titers to circulated strains have been shown in pair sera of ill patients in an HI test. The antibodies to virus proteins (HA) and internal proteins, nucleoprotein (NP) and matrix M1 protein, were also detected in these sera by Western blot investigations. The analysis of donor sera collected during 4 epidemic seasons in the Moscow region has revealed the dynamics of antibody level increases to modern strains from the beginning to the end of epidemic seasons. All modern strains were isolated in MDCK culture and most of them were non-sensitive to chicken embryonated eggs (CE) as regards their isolation from nasal swabs. The virus cultivations in CE after their isolation in MDCK were possible in middle 60, 3, 58% of cases for influenza A(H1N1), A(H3N2) and B viruses in 2003-2006. The

main differences in interactions between epidemic strains with human erythrocytes of both O(I), II blood groups and chicken ones were observed for influenza A viruses. IEEC conducts influenza surveillance in close cooperation with WHO Influenza centers in the USA and UK and 19 viral laboratories located in Russian regions. This work was supported in part by ISTC grant #3070.

5-009

### Ensuring effective maternity care during an influenza pandemic: findings of a report for the United Kingdom Department of Health.

**Bannister, B.<sup>1</sup>;** Sellwood, C.<sup>2</sup>

*<sup>1</sup>Department of Health, UK; <sup>2</sup>NHS London, UK*

Pregnant women are known to be susceptible to influenza and other viral infections due to the alterations in their cell-mediated immune responses, which protect the tissues of the placenta and fetus from rejection during gestation. To address the needs of this special group, the UK Department of Health (DH) formed an expert Working Group to identify key issues facing both providers of maternity services and the women and families who use them during a pandemic. The Group's organisers worked in close communication with the DH Maternity Matters group, which advises on the provision of a range of safe, accessible maternity services in the UK.

There are well-recognised issues common to the maternal health services of most developed countries (Rasmussen S.A., Jamieson D.J., Bresee J.S. Pandemic influenza and pregnant women. *Emerg Infect Dis.* 2008 Jan. Available from <http://www.cdc.gov/EID/content/14/1/95.htm>). These include planning for the provision of maternity services at a time of surge demand in healthcare, the safe use of countermeasures such as medicines and vaccines and ensuring effective infection control for pregnant women and their offspring while they are using maternity services.

The DH Working Group identified a number of additional issues of intense practical concern to pregnant women, their families and carers. Pregnant women have a special need for information about changes in the configuration of maternity services during a pandemic. They also require specific information on how to recognise possible influenza in themselves or in family members, where and how to get help, the use and safety of medicines and vaccines for themselves and their babies, and how to care safely for various family members whilst minimising risk to themselves.

Maternity care is heavily structured to include a sequence of screening tests to document the progress of pregnancy and the development of the fetus and allow for early counselling and intervention where problems are identified. Some of these tests,

such as nuchal scanning to detect Down's syndrome, may require expert interpretation and are only effective within a narrow time window. It may not be possible to guarantee such a structured schedule during a pandemic, so a process of prioritisation should be developed at the planning stage. This should take account of the risk of postponing some tests, using alternative screening methods (such as 'triple' blood testing), or even omitting some tests if they make a less critical contribution to the screening programme. Doctors, midwives and other carers must be prepared to explain this process, and to ensure that women whose pregnancy may be at special risk are offered appropriate screening wherever possible.

Pregnant women and their carers are usually anxious to avoid using medicines during pregnancy in case they have an adverse effect on the fetus or its development. Despite the widespread use in pregnancy of medications for conditions such as hypertension, diabetes, HIV infection and epilepsy, many women and their advisors may be unwilling to consider the use of medicines for treating influenza. There is accumulating evidence that fever of any cause can increase the risk of certain birth defects, and that treatment of fever with paracetamol can reduce this risk. Although paracetamol is accepted to be safe in pregnancy and for neonates, including premature babies, there is a reluctance to use it. Similarly, there may be anxieties about using antiviral or antibiotic treatment for influenza or its complications, although (with one or two well-recognised exceptions) there is no evidence of harm in using a wide range of anti-infective drugs. Specific information should be provided for women and midwives, so that they can make an informed decision about the high risks of influenza versus the low or absent risk from appropriate medication. Similarly, they should be made aware that influenza vaccines are now recommended for pregnant women in many countries, and could offer important protection to mother and fetus in a pandemic situation.

Planned arrangements for the birth may need to be altered at short notice, depending on the availability of staff and facilities. In particular, plans for a home delivery or use of a birthing pool may not turn out to be possible. Women need to understand and receive up to date information so that they can make flexible plans to allow for these possible changes.

Early return home after the birth is likely to be encouraged in a pandemic situation. Women will therefore need clear information on how to cope and where to get help if she or her new baby becomes ill. An example of immediate needs includes information about whether breastfeeding is possible or safe during an influenza illness.

Of approximately 460,000 UK women who are pregnant at any time, two thirds will already have at least one other child, and the majority will have a husband or partner with them at home. All of the above is an integral part of protecting other family members from influenza, protecting the woman from infection contracted within the family, and managing influenza illness in family members of different ages, for whom specific medications, doses and care arrangements may be needed. While the woman

herself may be concerned about managing the home and family if she develops influenza, a substantial part of this commitment is likely to be supported by her husband, partner or another family member. It is therefore important to think of the pregnant woman and her family as a unit when developing plans and advice for maternity care during an influenza pandemic.

5-010

### Surveillance of influenza cases presenting to emergency departments: a pilot study

**Bannister, Barbara**<sup>1</sup>; Reynolds, A.J.<sup>2</sup>; Gates, P.<sup>2</sup>; Wilcox, D.<sup>3</sup>; Millington, H.<sup>4</sup>

<sup>1</sup>Royal Free Hospital, UK; <sup>2</sup>Department of Health, UK; <sup>3</sup>ASCRIBE plc., UK; <sup>4</sup>Hammersmith Hospitals NHS Trust, UK

In an influenza pandemic, there would be an urgent need for up to date information about cases presenting for secondary care, the nature and severity of their illness and their treatment needs. Hospital statistics records are not completed in the UK until the patient leaves hospital, which may be long after first presentation, and pressures of the pandemic on staff and systems may cause further delay. Most acute admissions in the UK now pass through the hospital emergency department, where the case-presentation, clinical details and outcome (eg. admission, or release home) are immediately recorded electronically.

We piloted a system in which diagnoses and syndromes previously shown to be common in influenza cases presenting to emergency departments triggered a compulsory question: 'could this be influenza-related?'. A 'yes' answer led to a five question 'tick-box' asking whether antivirals and antibiotics were prescribed and whether influenza laboratory tests were performed. These replies were electronically collated with demographic and clinical information already present in the case-record, and used to provide a regular report of all identified influenza cases.

During the winter influenza season of 2007-8, 154 influenza cases were identified, with an epidemic curve corresponding to that described by the established primary care epidemic surveillance system. It was possible to document the age-range most affected, coexisting medical conditions, clinical investigations performed, data on numbers of prescriptions for antivirals and antibiotics, numbers of cases admitted to the hospital and the clinical area to where they were admitted.

This surveillance system places only a minimal extra burden on medical teams, and electronic reports could be produced up to daily in frequency. Further studies are now under consideration to test a wider system including representative hospitals covering all regions and administrations of the UK, to give a 'real-time' overview of the flu season as a surrogate of a pandemic wave.

5-011



## The community burden of Influenza and Influenza-Like Illness in England - early results from the MRC Flu Watch Study

**Kovar, Jana**<sup>2</sup>; Hayward, Andrew<sup>1</sup>; Warren Gash, C.<sup>2</sup>; Knott, F.<sup>2</sup>; Bermingham, A.<sup>3</sup>; Edmunds, J.<sup>3</sup>; Johnson, A.M.<sup>2</sup>; McMichael, A.<sup>4</sup>; Nazareth, I.<sup>5</sup>; Nguyen-Van-Tam, J.S.<sup>6</sup>; Watson, J.M.<sup>3</sup>; Zambon, M.<sup>3</sup>

<sup>1</sup>University College London, UK; <sup>2</sup>UCL Centre for Infectious Disease Epidemiology, UK; <sup>3</sup>HPA Centre for Infections, London, UK; <sup>4</sup>University of Oxford, UK; <sup>5</sup>MRC GP Research Framework, UK; <sup>6</sup>University of Nottingham, UK

**Introduction:** Seasonal influenza epidemics are responsible for substantial illness in the community, loss of economic productivity, general practice consultations, emergency hospital admissions and deaths. In the UK there has been a gradual decline in GP consultations for influenza-like illness (ILI) with low levels of reported consultations in recent years. Whether this is due to less illness or to changing modes of healthcare delivery or consultation patterns is unclear. We investigate the community burden of acute respiratory infections, ILI and laboratory-confirmed influenza in a community cohort of English households in 2006/07.

**Methods:** Households were randomly selected from lists of 43 general practices from the MRC GP research framework. At the time of recruitment (October-mid December 2006) blood samples were collected from adults and volunteers aged 5-16. Information on symptoms of respiratory illness was collected throughout the winter by weekly automated telephone calls and illness diaries. ILI was defined as fever  $\geq 38^{\circ}\text{C}$  plus either cough or sore throat. Spring blood samples were taken (March-April 2007) and the paired samples used to identify 4-fold rises in influenza antibody titre. We report findings from the winter of 2006/7.

**Results:** 607 individuals from 241 households participated. 43.2% reported at least one episode of respiratory illness (range 1-5 episodes). 25.9% had at least one ILI. There was a strong inverse relationship between illness frequency and age ( $p < 0.001$ ). 15.1% of participants seroconverted to influenza A H3N2 (the predominant circulating strain that season). Of those who seroconverted, 58% reported symptoms of which 72% met the definition of ILI. However, only 25% of those reporting an ILI seroconverted to H3N2, rising to 38% during the period of heightened national influenza activity. For those aged between 5 and 65 years, 21.9% of respiratory illnesses resulted in absence from work or school, rising to 26.9% for ILI and 35.5% for ILI in the influenza season. The mean absence (in those taking time off for ILI) was 2.4 days. Data on consulting and vaccination patterns will also be presented.

**Conclusions:** In this cohort, ILI and serologically-confirmed influenza were very common despite low levels of ILI consultations and flu incidence reported through national surveillance.

5-012

## Estimates of influenza incidence in France, season 2006-2007

**Vaux, S.**<sup>1</sup>; Le Strat, Y.<sup>2</sup>; Mosnier, A.<sup>3</sup>; Bensoussan, J.L.<sup>4</sup>; Valette, M.<sup>5</sup>; Enouf, V.<sup>6</sup>; Davidaud, I.<sup>3</sup>; Bonmarin, I.<sup>2</sup>; Grog, I.<sup>4</sup>; Lina, B.<sup>5</sup>; van der Werf, S.<sup>6</sup>; Levy-Bruhl, D.<sup>2</sup>; Cohen, J.M.<sup>3</sup>

<sup>1</sup>Institut de Veille Sanitaire (InVS) (French Institute of Public Health Surveillance), France; <sup>2</sup>Institut de Veille Sanitaire (InVS) (French Institute of Public Health Surveillance), Saint-Maurice, France; <sup>3</sup>Réseau des Grog, Paris, France; <sup>4</sup>Réseau des Grog, France; <sup>5</sup>National Reference Centre for Influenza (région sud), LYON BRON, France; <sup>6</sup>National Reference Centre for Influenza (Institut Pasteur), Paris, France

Providing accurate estimates of the influenza disease burden is complicated because many different pathogens cause infections with symptoms like those of influenza. The purpose of this study was to estimate the weekly incidence of true influenza cases in the community and to describe the temporal diffusion of the influenza epidemic according to age during the 2006-2007 influenza season in France. The study was based on a surveillance sentinel network (Grog) collecting, on a weekly basis, information on acute respiratory infections (ARI) and naso-pharyngeal swabs for laboratory confirmation during each influenza season. We established an active population-based surveillance based on a two-stage stratified random sampling design. The physicians were considered as a random sample representative of all general practitioners and pediatricians working in private practices in France regarding management of ARI cases. At the second stage, during the influenza epidemic period, each physician participating in the sampling protocol had to swab the first patient of the week presenting with ARI, belonging to a predefined age group and matching with criteria for swabbing. For each week, sampling weights were adjusted for post-stratification concerning the number of consultations and visits for practitioners participating in the study and for all practitioners with a liberal activity in France. During the 9-week long period of surveillance, 284 general practitioners and 77 paediatricians participated in the study. Among the 1266 swabs analyzed, 480 were positive for influenza, mainly A(H3N2), by virus isolation in MDCK cells. The incidence of consultations for laboratory-confirmed influenza was thus estimated to be 2.8/100 inhabitants (95% confidence interval: 1.8; 3.8). The highest influenza incidence was observed in the 5-14 year old group: 7.4/100 inhabitants (95% confidence interval: 4.6; 10.1), followed by the 0-4 year old group: 6.7/100 inhabitants (95% confidence interval: 3.4; 11.2), then by the 15-64 year old group and lastly by the over 64 year old group. Weekly estimations of incidence showed first increases for the 0-4 and 5-14 year old groups, then for the 15-64 year old group and lastly in the over 64 year old group. The proposed methodology allows for estimating the incidence of laboratory-confirmed influenza without intensive and long follow-up of a population required by costly cohort studies.



### Presentation of an infection control algorithm for use in healthcare facilities, ambulatory services and other emergency services in the event of pandemic influenza

**Gralton, Jan<sup>1</sup>**; McLaws, M.L.<sup>1</sup>; Rawlinson, W.<sup>2</sup>; Seale, H.<sup>3</sup>

<sup>1</sup>School of Public Health and Community Medicine, University of New South Wales, Australia; <sup>2</sup>Virology, South Eastern Area Laboratory Service, Prince of Wales Hospital, Australia; <sup>3</sup>School of Public Health and Community Medicine, The University of New South Wales, Australia

The increasing threat of an influenza pandemic has urged immediate review of pharmaceutical and non-pharmaceutical infection control measures for healthcare professionals and for those in the front line community services. Infection control practices include the appropriate use of personal protective equipment, social distancing measures, vaccination and antibiotic prophylaxis to prevent occupational acquisition of pandemic influenza. We have undertaken an evidence-based assessment of the literature for the infection control of SARS, avian influenza and pandemic influenza. Using guidelines set by the National Health and Medical Research Committee (Australia), our review analysed publications for levels of evidence given the inherent study design biases and confounding factors and the generalisability of the findings to the different healthcare professional groups. Consideration has also been given to the practicality and feasibility of implementing identified infection control practices as well as the compliance issues associated with each practice. The primary outcome of this assessment is our generation of an infection control algorithm based on evidence all the while considering practical and compliance issues. This algorithm will be employed in healthcare facilities and other emergency and community front line services. The resultant evidence-based infection control protocol also has potential for application in the wider community setting.



### Pyrosequencing as a tool to detect known markers of resistance to neuraminidase inhibitors in seasonal influenza A viruses

**Deyde, V.M.**; Okomo-Adhiambo, M.; Sheu, T.G.; Wallis, T.R.; Klimov, A.I.; Gubareva, L.V.

Influenza Division, Centers for Disease Control and Prevention, Atlanta, GA, USA

**Introduction:** In the United States, monitoring of resistance to two classes of drugs, adamantanes (M2 blockers) and neuraminidase inhibitors (NAIs), is conducted at the Influenza Division, Centers for Disease Control and Prevention, Atlanta, GA, which is the WHO Collaborating Center for Surveillance, Epidemiology and Control of Influenza. Previously, the pyrosequencing approach based on detection of established markers for adamantane resistance has been successfully utilized in monitoring resistance to M2 blockers in seasonal influenza A isolates. Resistance to the NAIs zanamivir and oseltamivir is currently monitored using the NA inhibition assay. The use of this assay as a primary method for monitoring resistance to NAIs requires virus propagation in cell culture prior to testing. Since resistance to NAIs is type/subtype specific, the NA inhibition assay also requires preliminary NA subtyping. Sequence analysis to identify genetic changes in NA of viruses demonstrating resistance phenotype is necessary to better understand mechanisms of NAI-resistance and their clinical relevance. In previous years, NAI-resistance among seasonal influenza viruses circulating in the communities has been very low (<1%). At the beginning of the 2007-2008 season, a considerable rise in resistance to oseltamivir was detected among A(H1N1) viruses circulating in different parts of the world, including North America, with the highest frequency seen in some European countries (up to 67%). The identified oseltamivir-resistant A(H1N1) viruses contained a H274Y amino acid change in the NA protein, which was previously detected in mutants that emerged in patients during oseltamivir treatment. The emergence and spread of oseltamivir-resistance among A(H1N1) viruses circulating in many countries, including the U.S., raises concerns regarding optimal treatment options available at this time for the control of influenza infections and necessitates detailed epidemiological investigations. In response to increased demands for enhanced drug resistance surveillance, we developed a pyrosequencing approach for detection of the 5 most commonly reported markers of NAI-resistance among seasonal influenza A viruses, including the H274Y mutation in the N1 enzyme.

**Methodology:** Full length NA sequences from 1200 human viruses collected between 1968 and 2008 were aligned. A consensus N1 sequence was generated and used for primer design using Pyrosequencing Assay Design software (Biotage). Similarly,

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a consensus sequence was generated based on alignment of 1300 NA sequences from human A(H3N2) viruses. Viral RNAs were extracted from A(H1N1) and A(H3N2) samples (138 clinical specimens and 36 viral isolates) using a MagNa Pure LC robot. RT-PCR amplifications were performed using the SuperScript™III One-Step HI FI system (Invitrogen). Primers were used at 20iM in a standard reaction mixture with 45 cycles of amplification. Codons and mutations are given in N2 subtype numbering.

**Results:** To validate the designed primers and the overall pyrosequencing protocol, a set of viruses that exhibit reduced drug-susceptibility in the NA inhibition assay was used. Their NAs contained previously described or new markers of NAI-resistance in N1: H274Y, and D151E; and in N2: E119V/I; D151N, A,V; R292K. The wild-type counterparts were used as sensitive controls. Using the pyrosequencing approach, detection of the most common mutation H274Y (CAT to TAT) that confers oseltamivir-resistance in A(H1N1) viruses was demonstrated with the use of the reference H274Y mutant of A/Texas/36/1991 and an additional 4 virus isolates from the current season. A noteworthy fact is that we were able to detect oseltamivir-resistant A(H1N1) viruses (H274Y) in the original clinical specimens. Among 161 original clinical specimens tested, 27 were found to contain the H274Y resistance marker while 134 did not. Therefore, the use of pyrosequencing provides a clear advantage over the chemiluminescent NA inhibition assay, which requires virus isolation and propagation in cell culture prior to testing for antiviral resistance. Furthermore, the pyrosequencing approach developed in this study also allowed for the detection of the following mutations in the N2: R292K (AGA to AAA), and E119V (GAA to GTA). A noteworthy fact is that pyrosequencing allowed detection of the mixture of V and I at residue 119 of the NA in a virus isolated from an oseltamivir-treated patient. Following plaque purification, we demonstrated that both clones, E119V and E119I, exhibited oseltamivir resistance in the chemiluminescent NA inhibition assay, thus revealing a novel mutation, E119I (GAA to ATA), associated with resistance to oseltamivir. Although the N294S mutant was not available for testing at this time, the designed primers for pyrosequencing allowed for testing codon 294 in N2 (AAC). The role of mutations at D151 in NAI-resistance seen in the NA inhibition assays remains unclear and clinical relevance has not been demonstrated. In the present study, we demonstrated our ability to detect changes at D151 in both N1 and N2 NA molecules by pyrosequencing. In one case, the change at position 151 was not detected in the original clinical specimen prior to virus propagation in cell culture although it was clearly present in the cultured virus isolate. This raises the issue of a possible selective role for cell culture in development of the NAI resistant genotype, which could be further explored with the use of the pyrosequencing technique.

**Conclusions:** We have designed a pyrosequencing assay for detection of established mutations that confer resistance to NAIs in seasonal influenza A viruses. High sensitivity of the assay allows detection of these mutations in clinical specimens thus reducing the time needed to obtain a result, which is an essential

factor in outbreak management and investigation. Additionally, this assay may be useful for detecting mutations arising as a result of virus passage in cell culture. The results of the assay are definitive in detecting established genetic markers of resistance and typically do not require further phenotypic testing. However, the pyrosequencing assay is not a replacement for the NA inhibition assays due to the limited knowledge on the genetic changes that confer resistance to NAIs.

5-015

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### The usefulness of annual proficiency testing for the diagnosis of influenza A H5 infections in humans

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Ellis, Joanna<sup>1</sup>; Curran, M.D.<sup>2</sup>; Wreghitt, T.J.<sup>2</sup>; Zambon, M.C.<sup>1</sup>

<sup>1</sup>Influenza Laboratory, Centre for Infection, Health Protection Agency, UK; <sup>2</sup>HPA, East of England, Clinical Microbiology and Public Health laboratory, Cambridge, UK

The creation in 2005 of a national H5 regional laboratory network, capable of rapidly and accurately detecting human H5N1 infections was an integral part of influenza pandemic planning response in the UK and the Republic of Ireland. The network was designed to increase the capacity and capability for molecular diagnosis of influenza, and provide a framework for escalating diagnostic capacity in the event of a pandemic. The quality assurance of diagnostic testing has been supported by the provision of CE marked, quantitated, positive control material, national Standard Operating Procedures, training programs, regular technical updates and monthly teleconference meetings. Annual proficiency testing has also been performed since 2005, to ensure that participating regional laboratories are accredited for H5 diagnostic testing. Proficiency testing has provided a means of ensuring that technical methods are regularly updated in view of rapidly changing laboratory environments, and assisted laboratories in troubleshooting.

The first H5 proficiency panel of simulated clinical samples containing high and low titred inactivated Clade 1 H5N1 viruses was distributed in October 2005 simultaneously to 20 laboratories in the UK, and the Republic of Ireland, and was arranged to arrive at each laboratory at the same time. The results demonstrated that all participating laboratories had established H5-specific assays in a 3–4 month period following a training programme. A clear confirmatory strategy was shown to be essential, and generic molecular influenza A/B diagnosis and subtype analysis could be used to enhance influenza surveillance programmes. Thirty-three percent of labs returned results within 6–8 hours. Further proficiency panels in 2006 and 2007 focussed on the detection of virus strains representing diverse circulating H5 lineages at clinically relevant concentrations, as well as other

subtypes (H1, H3, H7 and H9) and influenza B. All UK regional laboratories included in this exercise demonstrated the capability to detect and confirm diverse H5 strains, with improved delivery times of 6-8 hours by 88% and 94% of laboratories, in 2006 and 2007 respectively. Gaps in the ability to detect diverse avian strains were identified and reviewed.

Timed proficiency panel exercises are therefore an important and useful way of assessing laboratory diagnostic capabilities and testing technical decision-making in complex algorithms and clinical interpretation in outbreak investigations.

5-016

### The seasonality of influenza in the Asia-Pacific region

**Jennings, L.C.**

*(for the Asia-Pacific Advisory Committee on Influenza) Canterbury Health Laboratories, Christchurch, New Zealand*

The Asia-Pacific region is unique in that it spans a full range of climates, from Northern temperate, through tropical, to Southern temperate. In countries with temperate climates, both the periodicity and the seasonality of influenza virus infections are well established, and in these countries influenza is typically a winter disease, often with a clear peak in activity during the colder (winter) months. In countries in the tropical or subtropical zones influenza activity is either poorly defined or occurs throughout the year with periods of increased activity during the winter and rainy seasons; the seasonal pattern is generally less pronounced than in temperate zones and is only now becoming better defined following the introduction and increased intensity of surveillance programmes. The underlying cause of the seasonality of influenza remains unclear. Animal models have shown a direct effect of temperature and humidity on virus survival and transmission; however, epidemiological studies have failed to identify such an effect on transmission efficiency or host susceptibility to infection, or an indirect effect through behaviour changes in human crowding. Epidemic modelling suggests that the periodicity in incidence may be caused by undetectably small changes in the influenza transmission rate.

Following the formation of the Global Agenda for Influenza Surveillance and Control in 2002, the World Health Organization (WHO) has consistently encouraged countries to include the surveillance of influenza within their seasonal influenza control strategies as a priority on their public health agendas. Even though southern China has been recognized as the likely epicentre for the emergence of pandemic influenza for many years, surveillance activity in the region has been limited to the identification of circulating viruses in a few countries, and the epidemiology of influenza in many countries, especially those in the tropical and

subtropical zones, remains poorly understood. The importance of the south-east Asian region for the continuing evolution of seasonal influenza virus strains is now being established through the application of whole genome sequencing technologies and the availability of complete genome sequence data on viruses detected in geographically separated populations. Phylogenetic evidence strongly suggests that the temperate climate countries are seeded seasonally by influenza virus strains that evolve in tropical regions.

With the current level of global concern over the spread of the highly pathogenic avian influenza A(H5N1) virus and the threat of another human pandemic, the associated pandemic preparedness activities have allowed for the renewed focus on the laboratory and disease surveillance of influenza in the region. The WHO and Centers for Disease Control and Prevention (CDC) have established a coordinated approach to laboratory capacity building, the establishment of laboratory-based surveillance and surveillance networks. In every country where surveillance has now been established, influenza activity is being demonstrated. The comparison of surveillance data for influenza A/H1, A/H3 and B viruses over multiple years from 10 countries spanning the Asia-Pacific climatic regions highlights the continuous or near continuous circulation with rainy and dry season periods of increased activity in tropical and subtropical regions as well as the annual variation within each country and geographical variation. Clearly the seasonality of influenza needs to be established for individual countries if appropriate health policy for both the timing of influenza vaccine administration and vaccine composition (Northern or Southern Hemisphere) is to be developed.

5-017

### Influenza-like illness surveillance as an early warning system for H5 infections in Indonesia

**Sedyaningsih, Endang;** Soetiarto, F.; Roselinda, R.; Widoretno, W.; Subangkit, S.; Krisnanur, A.

*National Institute of Health Research & Development, MOH, Indonesia*

**Background:** In many developing countries, including Indonesia, influenza is not considered a serious community health problem. This is partly because of the existence of other more severe and prevalent infectious diseases, such as malaria, tuberculosis and AIDS, but also because there is little data of the burden of disease of influenza, as well as its epidemiological and virological characteristics. Since 1999, Indonesia has started to develop sentinel surveillance of influenza-like illness (ILI). Hospital and Health Center sites were added gradually in collaboration with international support. When the first human case of H5N1 in Indonesia was identified in July 2005, the ILI surveillance spearheaded

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efforts to detect H5 infections in human as early possible, complimented by the suspected H5 national referral system and sero-surveillance among communities at high risk.

**Methods:** The surveillance utilizes 48 sentinel sites across 22 of the 33 provinces in Indonesia. Throat and nasal swabs were collected from patients aged 1 year and older who met the case definition of ILI, and were sent to regional and central health laboratories. RT-PCR, viral isolation and culture, and luminex tests were performed to detect influenza A and B viruses, as well as other viruses. In the H5 referral system, throat and nasal swabs, and sera of H5 suspected patients were sent to regional and central health laboratories to be tested. Close contact investigations were conducted to collect epidemiological data and to detect additional cases.

**Results:** We highlighted the monthly distribution of patients with influenza-like illness between 2004 and 2007 in Indonesia. Influenza viruses were circulated throughout the year with type A showing a peak period of incidence in the rainy seasons (from November to January) while type B was more constant at lower prevalence throughout the year. Within influenza A, H3 sub-types were the ones which gave high fluctuations, while H1 were more constant. One case infected by H5 was also detected through this system. Enterorhino -, adeno-, and human meta pneumo viruses were some of the other viruses detected among the influenza negative ILI cases. The number of referred suspected H5 (avian influenza) cases rose from 248 cases in 2005 (July-December) to 635 cases in 2007. Approximately 80% of the suspected cases were subsequently found to be negative for influenza. Most of these suspected H5 patients were hospitalized for severe acute respiratory infection (SARI) cases. Among them, H5 and H3 were detected at similar proportions (7.7% and 7.6%), H1 3.9%, B 0.6% and A (untypeable) 2.2%. H5 cases were also reported higher in the rainy season. Similar to the ILI cases, other viruses were also detected among the influenza negative suspected H5 cases.

**Conclusion:** Influenza virus A and B are circulating in Indonesia all year long, with a peak of influenza A in the rainy seasons. H3, H1 and influenza B were detected from some of the acute severe respiratory tract infections in people who were suspected as H5 infected cases. In Indonesia where H5 is endemic among poultry and H5 human cases were detected regularly, it is very important to conduct ILI sentinel surveillance to be able to detect H5 infections as early as possible.

5-020

### Follow up serosurveillance in connection with detection of avian influenza in the UK, 2005-2008

**Hoschler, K.<sup>1</sup>; Kuhne, M.<sup>1</sup>; Brown, I.H.<sup>2</sup>; Rodrigues, B.<sup>3</sup>; Bracebridge, S.<sup>4</sup>; Morgan, O.<sup>5</sup>; Nair, P.<sup>6</sup>; Phin, N.<sup>7</sup>; Reid, J.<sup>8</sup>; Mason, B.<sup>9</sup>; Watson, J.M.<sup>7</sup>; Nguyen-Van-Tam, J.<sup>7</sup>; Zambon, M.C.<sup>1</sup>**

<sup>1</sup>Influenza Laboratory, Virus Reference Division, Centre for Infection, HPA Colindale, UK; <sup>2</sup>VLA-Weybridge, Addlestone, Surrey, UK; <sup>3</sup>Norfolk Primary Care Trust, Norwich, Norfolk, UK; <sup>4</sup>Regional Epidemiology Unit, HPA East of England, Institute of Public Health, Cambridge, UK; <sup>5</sup>Harrow Primary Care Trust, Harrow, Middlesex, UK; <sup>6</sup>Norfolk, Suffolk & Cambridgeshire HPU, Norfolk Office, Thetford, UK; <sup>7</sup>Respiratory Diseases Department, Centre for Infection, HPA Colindale London NW9, UK; <sup>8</sup>Cheshire & Merseyside HPU, Kirkby, Merseyside, UK; <sup>9</sup>National Health Service for Wales, South East Wales Health Protection Team, Cardiff, Wales, UK

After having been free of any form of avian influenza for more than a decade, highly pathogenic avian influenza (HPAI) virus has been identified on several occasions in mainland Britain between 2005 and 2008. Situations involving the identification of HPAI viruses have included a quarantine facility with imported exotic birds (2005), periodic identification in dead wild birds (2006/07/08), and two outbreaks in commercial poultry premises (2007). Viruses recovered from these individual events belonged to recently circulating Clade 2 H5N1 viruses. Extensive serological surveillance of subjects exposed to either infected quarantine or wild birds or infected poultry indicates extremely low risk of HPAI virus transmission to humans. None of the subjects, including those who were grouped into possible cases presenting with influenza like illness (ILI) or conjunctivitis, or fever and a much larger group of exposed asymptomatic individuals were found to have antibody to H5N1 as evaluated using microneutralisation (MN) and haemagglutination inhibition (HI) testing. These data, together with experience elsewhere could have implications for future risk management strategies following sporadic exposure to H5 HPAI. We suggest that follow-up serological surveillance could be focused only on those subjects that are symptomatic. In contrast, two outbreaks of low pathogenicity avian influenza (LPAI) virus H7N3 (2006) and H7N2 (2007) were associated with laboratory confirmed human infection. H7N3 virus was isolated from a farm worker with conjunctivitis and H7N2 virus was detected using RT-PCR, but not isolated, from four exposed individuals treated for ILI. Serological investigations demonstrated low levels of H7 antibodies in the conjunctivitis case from whom H7N3 virus was isolated. For 81 other subjects exposed to H7N3 no serological evidence of infection was found, including several subjects who had conjunctivitis. In the follow up investigation of the H7N2 outbreak no subjects with H7 antibodies were found. We conclude that localized and systemic infection with H7 viruses does not reliably result in a detectable serologic response measured by HI and MN despite confirmed viral detection, and clinical symptoms may not be predictive of

a serological response. These findings indicate the difficulty of attempting serological follow up of outbreaks of H7 infection and point towards the necessity to develop assays with increased sensitivity for detection of H7 infection.

5-021

### Estimation of influenza-associated mortality in South Korea and the United States: a comparative study

Kang, Jong-Won<sup>1</sup>; Miller, M.<sup>1</sup>; Viboud, C.<sup>1</sup>; Lee, S.W.<sup>2</sup>

<sup>1</sup>NIH, Fogarty International Center, USA; <sup>2</sup>Korea CDC, Republic of Korea

**Background:** Similarly to other temperate countries, mortality in South Korea increases sharply during winter, which is plausibly associated with influenza virus activity. However there has been no quantitative study of influenza-associated mortality so far in this country. We estimated influenza-associated mortality among South Korean seniors over 65 years of age between 1998 and 2006 and compared with similar estimates for the USA.

**Methods:** We compiled age-specific monthly mortality for South Korea, 1998-2006, and for the USA, 1998-2004, for three senior age groups (65-74, 75-84 and 85 or over) and three disease categories: pneumonia and influenza (P&I), all-respiratory diseases (AR) and all-cause (AC). Influenza-associated seasonal mortality was estimated for each age group and death categories by a Serfling regression approach, as mortality in excess of a seasonal baseline, during periods of influenza activity. Periods of influenza activity were defined by applying a Serfling approach to monthly deaths coded specifically as influenza.

A cruder empirical method was also considered, where excess mortality was calculated by subtracting December-March mortality rates and November rates. The empirical method provided an upper limit on seasonal estimates of excess mortality rates, especially in seasons with no or very short periods of influenza activity, as defined by influenza-specific mortality. Excess mortality rates were calculated for each disease category, winter season, age group, and country and averaged across epidemic seasons.

We also computed the proportion of winter deaths attributable to influenza, as the ratio of influenza-associated excess deaths to deaths occurring in December-March. Data on influenza-like-illness (ILI), and viral surveillance were used to confirm periods of influenza activity and identify the dominant subtype each season.

**Results:** Influenza-specific mortality increased in 4 of the 8 winter seasons studied in South Korea, and in 5 of 6 seasons studied in the USA. Crude P&I mortality rates in Dec-Mar in South Korean seniors were about half those of the USA, and a similar ratio was observed in crude summer rates. Korean AR mortality in South

Korean seniors was about 60-80% of that of the US, while AC mortality rates were similar in the two countries.

Excess P&I mortality rate estimates based on the Serfling approach were systematically lower in South Korea, as compared with the USA (ratio of P&I excess mortality between the two countries, 0.4 to 0.5, depending on the age group). The difference was reduced by estimating excess mortality from broader death categories (ratio of AR excess mortality, 0.7 to 1.2, and ratio of AC excess mortality, 0.7 to 0.9). By contrast, there was little systematic difference in the proportion of winter deaths attributable to influenza between the two countries, for any death categories (ratios between 0.7-1.2).

The empirical method generally produced similar estimates than the Serfling approach for South Korea, especially for older age groups and broader death categories. In the 4 seasons with increased influenza-specific mortality, 10-35% of mortality recorded during Dec-Mar was attributed to influenza, depending on the death category. In the 4 seasons with no increase in influenza-specific mortality, only 2.2-7.4% of Dec-Mar mortality was attributed to influenza. Analysis of laboratory surveillance data for South Korea showed substantial A/H3N2 viral activity for all 8 seasons studied, except the 2001/02 season, in which H1N1 and B were dominant. However, both the Serfling and empirical excess mortality approaches suggested no or minimal excess mortality in the 2001/02, 2003/04, 2004/05 and 2005/06 seasons in this country.

**Conclusion:** The Serfling regression method proved to be applicable to South Korean mortality data, especially when applied to broad death categories such as all respiratory or all-cause mortality. Crude and excess P&I mortality rates were substantially lower in South Korea than in the USA, while excess AR and AC were more comparable. The proportion of winter deaths attributable to influenza was the most comparable indicator of influenza burden between countries. Taken together, this suggests under-utilization of P&I code in Korea; in this situation, AR can be a more useful disease category than P&I.

Four recent winter seasons were associated with low or no influenza excess mortality in South Korea, despite concomitant isolation of influenza virus and reports of visits for influenza-like-illnesses. This period of low excess mortality coincided with the introduction of a nationwide influenza vaccination program in 2002, which generated very high coverage among the elderly (80% in 2005). Further studies are required to quantify the impact of vaccination on influenza-associated mortality among South Korean seniors.

5-022

# **The feasibility of reporting daily surveillance data during an influenza pandemic: report from a United Kingdom exercise**

**Mook, P.<sup>1</sup>; Reynolds, A.<sup>2</sup>; Mobsby, V.<sup>3</sup>; Conroy, S.<sup>4</sup>; Chamberland, M.<sup>1</sup>; Phin, N.<sup>1</sup>; Watson, J.M.<sup>1</sup>; Gates, P.<sup>2</sup>; Simpson, J.<sup>3</sup>; Barker, M.<sup>3</sup>; Joseph, C.A.<sup>1</sup>**

<sup>1</sup>Centre for Infections, Health Protection Agency, UK; <sup>2</sup>Department of Health, UK;

<sup>3</sup>Centre for Emergency Preparedness and Response, Health Protection Agency, UK; <sup>4</sup>Civil Contingencies Secretariat, Cabinet Office, UK

**Introduction:** Surveillance of influenza at national and global levels will be critical during an influenza pandemic. To date there has been limited evaluation of how this would operate in practice. It is anticipated that there will be a demand for a variety of influenza activity indicators reported as frequently as daily during a pandemic. To ensure that the United Kingdom (UK) (England, Scotland, Wales and Northern Ireland) surveillance strategy will meet the information needs of those involved in the pandemic response, major steps, including harmonising surveillance schemes in the four countries of the UK where possible, developing capacity for daily reporting and conducting a number of preparedness exercises, have been taken over the last three years. The Department of Health (DH) commissioned the Health Protection Agency (HPA) Centre for Emergency Preparedness and Response to develop a UK-wide exercise (United Endeavour II) to test the feasibility and processes of providing a daily influenza surveillance report to the DH and the Civil Contingencies Secretariat (CCS) of the Cabinet Office, which serves as a central point of co-ordination for government departments during national emergencies.

**Methods:** Influenza surveillance schemes in England, Scotland, Wales and Northern Ireland routinely report data on a weekly basis throughout the influenza season to the HPA Centre for Infections (CfI). For the purpose of the exercise, these surveillance schemes were requested to submit daily and weekly data to CfI for the period of 14 - 18 January 2008. Data were submitted electronically using a DH-developed password protected informatics website. CfI collated these data to generate a daily report for the DH that described and interpreted the national epidemiological situation. Prior to the exercise, a standard report template was agreed for both the daily and weekly reports. The daily report template included sections to report General Practitioner (GP) consultation rates for influenza-like illness (ILI), death registrations due to all causes, virological strain and antiviral susceptibility data, outbreak reports and the proportion of calls for cold/flu and fever to 24-hour nurse-led National Health Service (NHS) telephone help lines (one for England and Wales and one for Scotland). The weekly report template included additional clinical and antimicrobial susceptibility data and a weekly figure of registered deaths. The DH combined these surveillance data with additional NHS hospital operational data to produce a central health report

for the CCS. Each of these process steps had an associated daily deadline. This exercise was evaluated in terms of the timeliness, quality and resilience of both the data submission process and staff requirements and usefulness of the outputs.

**Results:** With a few exceptions, the daily surveillance data and report submissions met the exercise deadlines. A DH health report was submitted each evening to the CCS. While the majority of surveillance schemes were able to collect and report new daily figures, two of the GP schemes could only report weekly figures and the telephone help line in England and Wales was unable to report any data due to a technical data access problem that was not resolved until after the exercise. In addition, only about half of the local register offices in England and Wales were able to report daily death data centrally because a new electronic reporting system had not yet been fully implemented.

Resilience of the data submission process was tested when, as part of the exercise, the password protected informatics website was taken offline without advance warning for one day. All of the surveillance schemes followed the pre-agreed resilience plan and successfully submitted data by e-mailing CfI. Although staff resilience was not a problem during a five-day exercise, participants acknowledged that sustainability throughout a pandemic would be challenging.

Several areas for improvement were identified. Further work is needed to assess if a seven-day rolling ILI average rather than a daily ILI rate provides a more useful picture of the level and trends of influenza. To facilitate interpretation of data at the UK level, harmonisation of key definitions and time frames across the four countries should be sought whenever possible. For example, in the weekly report, the lag time between the week of death registration and report varied from 1 to 2 weeks among the different countries. Also, countries released weekly death data on different days. The daily and weekly report template that was used to submit data to DH was overly-detailed and could be simplified and re-formatted. The CCS identified the need for a more "user-friendly" and succinct summary of surveillance data utilizing graphical displays whenever possible.

**Conclusion:** Exercise United Endeavour II demonstrated that providing a daily influenza surveillance report was achievable but will require improved staff resilience. The password protected informatics website facilitated submission, management and reporting of key surveillance data. The exercise further highlighted the critical importance of resilient IT systems. Presentation of the wealth of data from multiple surveillance schemes in the UK needs to be in a format that is easily understood. This is even more important when, during a pandemic, surveillance data will be supplemented with modelled estimates of cases and deaths, adding an extra complexity to interpreting a daily report.



### Application of social network analysis to characterize poultry movement in Cambodia and the possible spread of HPAI/H5N1 across the network

**Van Kerkhove, M.D.<sup>1</sup>; Vong, S.<sup>2</sup>; Holl, D.<sup>3</sup>; Guitian, J.<sup>4</sup>; Mangtani, P.<sup>1</sup>; San, S.<sup>5</sup>; Ghani, A.<sup>6</sup>**

<sup>1</sup>Infectious Disease Epidemiology Unit, London School of Hygiene and Tropical Medicine, UK; <sup>2</sup>Institut Pasteur du Cambodge, Cambodia; <sup>3</sup>National Veterinary Research Institute, Department of Animal Health and Production, Cambodia; <sup>4</sup>Epidemiology Division, The Royal Veterinary College, UK; <sup>5</sup>National Veterinary Research Institute, Department of Animal Health and Production, Cambodia; <sup>6</sup>MRC Centre for Outbreak Analysis & Modelling, Department of Infectious Disease Epidemiology, Imperial College London, UK

**Background:** H5N1 is believed to be endemic in domestic poultry in Cambodia and has been circulating in SE Asia since late 2003. The movement of poultry through markets is potentially important in the circulation and spread of HPAI/H5N1 virus throughout the region, yet little is understood about the poultry market chains in Cambodia, the frequency with which backyard poultry enter the market chain and the efficacy of potential interventions to control the spread of H5N1 along the poultry network.

**Methods:** A cross-sectional survey of rural Cambodians, rural, peri-urban and urban market sellers and middlemen was conducted in Phnom Penh and six Provinces to evaluate weekly and seasonal poultry movement and selling practices throughout Cambodia and to highlight where interventions need to be targeted in the case of H5N1 outbreaks among poultry. Qualitative methods, including focus group discussions and in-depth interviews with provincial and district veterinarians, were used to identify markets and middlemen. Standardised structured questionnaires were given to village chiefs, heads of households, market sellers and middlemen using four separate validated questionnaires by trained Cambodian interviewers. With empirical data on weekly and seasonal poultry selling and transport patterns, a directed network of chicken and duck movements within and into Cambodia was analyzed.

**Results:** A total of 115 village chiefs, 600 heads of households (100 from each study province) from 115 randomly selected villages, 139 middlemen and 123 market sellers were recruited and interviewed. A total of 113 poultry markets were identified in the study areas, including 43 markets in Phnom Penh. All of the identified markets in Phnom Penh sell prepared (boiled and de-feathered) poultry and almost half are wet markets (46.3% sell live poultry and slaughter on premises). Few rural Cambodians reported selling chickens (3.8%) or ducks (0.5%) outside of their home village during the previous eight-month period. Poultry is predominately transported throughout and into Cambodia by middlemen. The source of poultry was identified from nine

Cambodian provinces, however cross-border trading of chickens and ducks from Vietnam and Thailand was identified. The destination of most poultry movement (84.3%) via middlemen is >10 km from the source and directed into Phnom Penh (markets and farms supplying markets). A substantial increase in poultry movement via middlemen and market sellers was found just prior to national holidays (i.e., Chinese New Year and Khmer New Year).

**Conclusions:** Most poultry movement occurs into Phnom Penh making the markets in Phnom Penh a potential hub for the spread of H5N1 and ideal for surveillance and control. Poultry movement within the Province bordering Thailand and into this Province from Thailand is separated from the main network linking Phnom Penh; however poultry from Vietnam were linked to the main network and Phnom Penh. Domestic poultry outbreaks of H5N1 have occurred in areas of the main network and therefore Phnom Penh markets, namely wet markets, are best for routine surveillance activities and control interventions.

5-024

### Safety and tolerability of a virosomal adjuvanted influenza vaccine (Inflexal® V): an analysis of spontaneous cases reported over 11 influenza seasons

**Schenk, N.; Hartmann, K.; Koller Doser, A.**

Crucell Pharmacovigilance Department, Crucell, Berna Biotech Ltd, Berne, Switzerland

**Background:** Manufacturers are required to conduct adequate ongoing monitoring and benefit/risk evaluation during the post-authorization period with the aim of ensuring that safety hazards are minimized and benefits maximized by appropriate action. The cornerstone of the continuous surveillance of safety and risk-benefit ratio of marketed products are spontaneous reporting systems, i.e. passive surveillance systems which rely on health professionals, vaccinees, or others to voluntarily submit reports of illness or reactions following vaccination, termed spontaneous adverse drug reaction (ADR) reports. These systems can generate signals of potential safety problems that must be tested through more rigorous epidemiologic methods. Spontaneous ADR reports can additionally provide important information on at-risk groups, risk factors, clinical features of known serious ADRs, or can support lot surveillance.

**Objective:** To evaluate the safety and tolerability of a virosomal adjuvanted influenza vaccine (Inflexal® V) and to identify potential new risks through in-depth analysis of spontaneous ADR reports submitted to Crucell's Pharmacovigilance Department (CPD) from worldwide sources over 11 influenza seasons.

**Methods:** All spontaneous ADR reports were analyzed and

classified according to the type of adverse reaction, such as injection site reactions, systemic vaccine reactions, neurological and allergic / hypersensitivity reactions. Based on these reports, an ADR profile was constructed and reporting rates for the respective ADR types were calculated, based on distributed doses.

**Results:** In the 11 influenza seasons from 1997 to 2008, a total of 389 spontaneous ADR cases were reported to CPD, detailing 694 adverse events (AEs). Systemic vaccine reactions accounted for 1/3 of all reported AEs, followed by allergic / hypersensitivity reactions (15%), injection site reactions (13%), neurological reactions (12%), and vascular disorders (3%). Reporting rates per 100,000 doses distributed were, according to the type of reaction, 0.2 for injection site reactions, 0.6 for systemic reactions, 0.2 for neurological reactions, 0.25 for allergic/hypersensitivity reactions, and 0.06 for vascular disorders. The overall reporting rate was 0.95 cases per 100,000 doses distributed. The most commonly reported systemic reactions were fever (>38°C), muscle / joint pain and headache; the most commonly reported local reactions were redness and swelling at the injection site. The vast majority of these cases were non-serious and resolved within a few days. Ten cases of a suspected Guillain Barré syndrome (GBS) associated with Inflexal® V were reported. This results in a GBS reporting rate of 1 case per 4.1 million doses distributed or 0.024 cases per 100,000 doses distributed. These numbers are far below the natural median incidence rate of 1.3 cases per 100,000 person/year (range 0.4-4.0). Overall, 6 fatal cases have been reported following Inflexal® V vaccination during these 11 influenza seasons (mean age 75.6 years); in all cases causality was unrelated to the vaccine.

**Conclusion:** The results of an in-depth analysis of the safety and tolerability data for Inflexal® V using spontaneous cases reported to CPD from worldwide sources during 11 influenza seasons (1997 to 2008) supports the safety and high local tolerability of Inflexal® V. The overall reporting rate of serious and non serious ADRs is low, and no new significant risks were identified in these 11 influenza seasons. The current benefit/risk evaluation remains favorable.

5-025

### Risk communication of influenza like illness for health providers in Indonesia as a part of pandemic preparedness

Farida, Soetiarto<sup>1</sup>; Farida, S.<sup>2</sup>

<sup>1</sup>National Institute of Health Research & Dev., Indonesia; <sup>2</sup>NIH-RD, Indonesia

**Background:** Avian Influenza (AI) in humans ranges from typical human Influenza-Like Illness (ILI) to pneumonia. Indonesia ranks highest in the world up to March 31, 2008 with 132 cases

of AI and 107 deaths. More cases are to be expected since the influenza A (H5N1) viruses are circulated among poultry in most of the 33 provinces in Indonesia. A good pandemic preparedness plan has to be supported by the whole community and not just by the government. To increase awareness and to motivate the community, a good plan in risk communication is needed, especially in a vast geographical country like Indonesia. As part of the national preparedness plan, the Government of Indonesia has taken some steps e.g. in 2006, strengthening the ILI surveillance system in 20 sites of 10 provinces, and in 2007, developing risk communication materials for health providers for ILI for provinces participating in the sentinel surveillance system. Risk communication has an important role in preventing and as an early warning of the pandemic of human A.I. The objectives are: 1. To increase knowledge on ILI of the health professionals. 2. To empower health providers as the main communicators to their patients on the signs of ILI. 3. To increase risk-awareness of ILI and A.I among health professionals and their clients.

**Methods:** Firstly, the message & materials are developed. Secondly, qualitative research is conducted among the health providers and patients to measure the level of knowledge and awareness of ILI symptoms and to test the message. Thirdly, training for trainers (TOT) of Provincial Health Officers, District Health Officers, Medical Doctors of Public Health Centers and Public Hospitals from each province.

**Results:** Qualitative research findings showed that the health providers had little awareness about ILI; ILI was regarded as common flu, not a dangerous disease; however, they perceived that the most dangerous flu was AI. Health professionals referred to time limitation as the main cause of poor communication practices. Clients will visit health providers only if the symptoms are not cured by OTC medicine. Clients talked about avian flu only when exposed by media, they would ask about the possibility of them and their families getting the diseases. This was a rare event. Based on the qualitative results, the contents of the message for the patients to reduce transmission followed Flu-WISE behaviors: Wash: regular hand washing, Inform: seek & share information about ILI, Separate: keep distance from crowds when sick with flu. Etiquette: cover mouth and nose with tissue when sneezing or coughing; (adapted to the Indonesian language). We developed ILI standing banners for display at Health Centers & Hospitals, a poster with a version for kids and grandmothers, a leaflet & 2008 calendars for patients, and a guidebook for health providers containing information on technical aspects of medical intervention advice. TOT for influenza care was conducted for health professionals at ILI surveillance sites. Serial assessments of the available 9 modules were conducted at the start and end of the courses. In general, knowledge and skills increased through the courses. For example, the correct answers for the overview increased: from 81.25%, to 99.0% respectively; training approach: from 69.23% to 98.4%; health promotion: from 51.20% to 98.7%; communication skills: from 80.76% to 99.0%; creating a positive learning atmosphere: from 71.79% to 97.5%; interactive presentation: from 58.33% to 100%; facilitating skills:

from 85.89% to 100%; coaching: from 66.66% to 100%; and plan for training: from 62.30% to 92.2%.

**Conclusion:** Risk communication is a critical part of the pandemic preparation plan, more training must be conducted involving more people. Further evaluation is needed to ensure that risk communication is carried out by health providers and that the messages are implemented by the community. [The project was funded by CDC Atlanta].

5-026

### Perception and coverage of influenza vaccine among pediatric nurses and pediatricians

Nitsch-Osuch, Aneta<sup>1</sup>; Dyk, S.<sup>2</sup>

<sup>1</sup>Department of Family Medicine, Medical University of Warsaw, Poland;

<sup>2</sup>Medicover Center, Poland

**Background:** Health care workers should be routinely and annually vaccinated against influenza for epidemiological reasons. Persons who take care of children aged 0-59 months belong to the same group because of the same indications. Pediatric nurses and pediatricians have at least a double reason to be vaccinated – being health care workers and having contact with infants and young children.

**Aim:** The aim of the study was to determine general perception and coverage of influenza vaccine among pediatric nurses and pediatricians in a network of private ambulatory care units.

**Material and methods:** The self-administrated survey was completed by 128 nurses, of whom 66 were pediatric nurses and 104 doctors, of whom 47 were pediatricians. The survey was validated (kappa score 0.8). The median age of nurses was 34 years (SD 2.4), while the median age of doctors was 44 years (SD 4.2).

**Results:** Among pediatric nurses, 43% performed influenza vaccination occasionally, 18% annually, while 39% avoided this vaccination. Among paediatricians, 58% performed influenza vaccination occasionally, 24% annually, while 18% have never had this vaccination. Most doctors declared they strongly recommend influenza vaccination to their patients and their families (74%). Annual vaccination against the flu was found to be a factor that might have influenced the frequency of vaccination among patients (OR 3.2; 95% CI 2.6-3.7). Many doctors and nurses declared they would plan the influenza vaccination in the future (respectively 54 and 48%). The main reason for not having vaccination among examined medical staff was: fear of the possible side effects (48% and 35% respectively among nurses and doctors), disbelieving the effectiveness of the vaccination (32% and 53%), and perceiving influenza as a mild disease not requiring prevention (20% and 12%). Among

nurses and pediatricians who performed vaccination annually, 82% did it because of the willingness to avoid the illness and its complications, 8% because they belonged to a risk group due to a medical condition, and 1.5% because of the willingness to protect patients.

**Conclusions:** The influenza vaccine coverage among pediatric nurses and pediatricians was generally low. The need for vaccination against influenza among health care workers should be strongly emphasized.

5-027

### Age specific influenza mortality in the Czech Republic in 1986-2006: the need to focus on the most vulnerable groups

Kyncl, J.<sup>1</sup>; Prochazka, B.<sup>2</sup>; Havlickova, M.<sup>1</sup>; Vit, M.<sup>3</sup>

<sup>1</sup>Centre for Epidemiology and Microbiology, National Institute of Public Health,

Czech Republic; <sup>2</sup>Dept. of Biostatistics, National Institute of Public Health, Czech

Republic; <sup>3</sup>Division of Chief Public Health Officer, Ministry of Health, Czech Republic

**Background:** Respiratory virus activity is detected in Europe each winter, yet the precise timing and magnitude of this activity remains highly unpredictable. Influenza infection is often underestimated, being easily mistaken for one of many acute respiratory infections (ARI) as it shows similar clinical symptoms. There is still low influenza vaccine uptake in the Czech Republic (approximately 10% of the population during the 2006/2007 season). The aims of this study are to find correlation between mortality and influenza morbidity and to model mortality in different weeks of the year outside the influenza epidemic.

**Methods:** Data on daily deaths from all causes and deaths from diseases of the circulatory system in the Czech Republic were available for influenza seasons 1986/87 - 2005/06 (altogether 2 317 522 and 1 267 718 deaths reported, respectively) and for eight age groups (0, 1-4, 5-14, 15-24, 25-59, 60-69, 70-79, 80+ years). Data on the incidence of influenza and other ARI were taken from the surveillance programme. The weeks in which ARI morbidity exceeded the epidemic threshold and at the same time, circulation of influenza virus among the population was reported by the National Influenza Centre, were considered as influenza epidemic weeks. Analysis was based on the assumption that outside the epidemic periods, deaths are distributed according to the Poisson distribution with a linear trend depending on time and with periodic behaviour during the year. The morbidity rate is only expected to increase in the epidemic compared to non-epidemic period.

**Results:** When comparing the weekly morbidity from acute respiratory illnesses and weekly mortality for all causes of death, the peaks of these two parameters almost overlap. In the epidemic

period (152 weeks) 27.6% of findings were above the unilateral 95% tolerance limit of the model, compared to the non-epidemic period (926 weeks) with only 4.3% of findings above this limit. The mean estimated excess of annual deaths from all causes was 1914 (18.58 per 100 000 population). The median of deviations of the estimated number of deaths from the actual number of deaths was negligible at 11.1 (95% CI = (-2.7; 20.9), stat. NS) for the non-epidemic period, being equal to 133.6 (95% CI = (88.2; 172.6),  $p < 0.001$ ) for the epidemic period. Analysis of the age specific excess of deaths showed wide variation between age groups from 4.3 per 100 000 population in the 15-24 year olds to 370.5 per 100 000 population in the 80+ year olds, respectively. Similar results were found for deaths from diseases of the circulatory system accounting for 54.7% of all deaths in the study period. The median of deviations of the estimated number of deaths due to diseases of the circulatory system from the actual number of deaths is 7.1 for the non-epidemic period (stat. NS), being equal to 83.4 for the epidemic period,  $p < 0.001$ .

**Conclusions:** The presented results confirm clearly and unambiguously the excess in death rates during the influenza epidemic periods, depending on the duration and magnitude of the epidemic. The mean annual excess rate for the Czech Republic is 1.7 % of the population, the major part of this rate being attributable to influenza. The number of influenza excess deaths dramatically increases after the age of 60, with the highest rate recorded in the elderly group (80 years and older). Vaccination against influenza proved both effective and cost-effective and therefore is to be recommended as the most important preventive measure.

5-028

## A tale of two cities: influenza in Southern China

Yang, L.<sup>1</sup>; Chan, K.P.<sup>1</sup>; Chen, P.Y.<sup>2</sup>; He, J.F.<sup>3</sup>; Peiris, J.S.M.<sup>4</sup>; Wong, C.M.<sup>1</sup>

<sup>1</sup>Department of Community Medicine, The University of Hong Kong, China;

<sup>2</sup>South Medical University, China; <sup>3</sup>Guang Dong Center of Disease Control, China;

<sup>4</sup>Department of Microbiology, The University of Hong Kong, China

Tropical and subtropical regions have been regarded as potential reservoirs for influenza viruses, but studies on disease burden and transmission patterns of influenza are lacking in these regions, largely due to the unpredictable seasonality of influenza and limited surveillance systems. It is particularly critical to understand the transmission pattern of influenza viruses in Southern China, which has been regarded as a potential epicenter for influenza pandemics, in order to efficiently contain such pandemics. In this study we took advantage of long-established influenza surveillance systems in two big cities of Southern China: Hong Kong and Guangzhou, to explore the transmission

pattern of influenza between these two cities. Both cities have crowded living conditions and similar subtropical climates (only one hundred miles apart), but Hong Kong is more economically developed compared with Guangzhou. Given that Hong Kong is an international travel hub, we hypothesized that there would be a directionality of transmission between these two cities. Influenza virus activity was measured by weekly proportions of specimens positive for influenza viruses which were collected from the laboratory surveillance in both cities. During the years 2004-2006, influenza virus activity exhibited an annual cycle in both Hong Kong and Guangzhou; while in 2003, influenza in Hong Kong showed a semiannual cycle (one peak in the winter and another in the late spring), but only one peak (in the early spring) could be identified in Guangzhou. We adopted phase analysis to estimate the synchrony of influenza virus activity between these two cities. The results showed that the oscillation of influenza virus activity in Guangzhou lagged behind that in Hong Kong by an average of 2.2 weeks in the year 2003, then surpassed that in Hong Kong in 2004, leading by an average of 1.1 weeks during 2004 and 2005. Influenza virus activity in Guangzhou lagged behind that in Hong Kong again in 2006, by an average of 0.4 weeks. These results suggest that influenza virus activities are synchronous between Hong Kong and Guangzhou, but there is no consistent directionality for transmission of influenza between these two cities. The mechanisms underlying the differences in transmission direction need further investigation.

5-029

## Pacific Regional Influenza Pandemic Preparedness Project

Ammon, C.<sup>1</sup>; Kiedrzyński, T.K.<sup>2</sup>

<sup>1</sup>Secretariat of the Pacific Community, Switzerland; <sup>2</sup>Secretariat of the Pacific Community, French Southern Territories

Secretariat for the Pacific Community (SPC) BP D5, 95 Promenade Roger Laroque, Anse Vata 98848 Noumea Cedex, New Caledonia Tel.: +687 26.20.00 Fax: +687 26.38.18 Catherinea@spc.int, Tomk@spc.int.

The Pacific Regional Influenza Pandemic Preparedness Project (PRIPPP) is designed to build the capacity of Pacific Island Countries and Territories (PICTs) to deal with the potential threat of another pandemic and other emerging diseases. Its goal is to have PICTs able to effectively and efficiently respond to emerging diseases, in particular highly pathogenic avian influenza (HPAI) and pandemic influenza, in line with regional and international guidelines and regulations. Its objectives are to implement immediate measures to prevent, or respond to, a possible outbreak of HPAI and pandemic influenza and to build sustainable capacities to respond to emerging diseases. Approximately 9.2 million people live in 22 PICTs spread over the Pacific Ocean,

covering 30 million square kilometres. They are commonly grouped into 3 subregions, Micronesia, Melanesia and Polynesia. The populations live in both medium and high density urban areas as well as in remote, sparsely populated areas, islands and atolls, often in close proximity to animals including poultry. Three broad areas of intervention are considered: 1) Preparedness and emergency plans, 2) Surveillance and response by public and animal health systems, and 3) Regional coordination and project management. The major constraints and challenges in the PICTs the limited resources, e.g. human or technical, and island isolation. The needs identified by the PICTs are to (i) review, accelerate, and finalize the national/territorial planning and test the plan through a real-time exercise; (ii) have a multi-sectoral process, (iii) carefully plan border control measures, (iv) address other public health measures before considering antivirals and pandemic vaccines, given the uncertainty of effectiveness, high cost and limited availability of these interventions, and (v) develop a surveillance system sufficiently sensitive to detect any unusual cluster of influenza-like illness. PRIPPP is a four-year project (2006-2010) totalling nearly AUD 12 million in addition to activities funded by USA (CDC) and ADB, with the support of its major donors Australia, New Zealand and France in partnerships with FAO, OIE, WHO and UNICEF. It employs around twenty staff.

5-030

### The effectiveness of telephone help line and A&E data as an influenza-specific syndromic surveillance strategy for the Province of Ontario

**Rolland, Elizabeth<sup>1</sup>**; Moore, K.M.<sup>2</sup>; Mangtani, P.<sup>1</sup>

<sup>1</sup>London School of Hygiene and Tropical Medicine, UK; <sup>2</sup>Queen's University Public Health Informatics, Canada

**Background:** Syndromic surveillance systems (SSS) such as telephone helplines (Telehealth in Ontario is one such helpline) are increasingly being evaluated as complementary surveillance systems, in that they can provide prediagnostic data to rapidly detect infectious disease outbreaks before they are detected through conventional surveillance methods (often in real-time). No research has been done on the usefulness of A&E or Telehealth as an SSS in Ontario.

**Description of Data:** The Ontario Telehealth System ("Telehealth") is a toll-free telephone helpline provided by the Ontario Ministry of Health and Long-term Care, and is available to all residents of Ontario. Telehealth operates 24 hours a day, 7 days a week and all calls are answered by registered nurses who have at least three years of recent clinical experience. For all medical calls the call nurse follows through a decision tree, based on the algorithm that the nurse assesses as best describing the caller's initial

complaint. The 440 algorithms available to the call nurse were classified by an emergency department physician into 6 revised Real-Time Outbreak Detection System (RODS) prodromes. The calls with algorithms classified in either the respiratory or fever/ILI prodromes are the ones used in this evaluation.

The National Ambulatory Care Reporting System (NACRS) tracks all ambulatory care (A&E, drop-in, day surgery) visits that take place in Canada, and is collected by the Canadian Institute for Health Information (CIHI). All Ontario hospitals are required to provide information to CIHI on patient visits on a regular basis, using the most recent version of the Canadian Enhancement to the International Statistical Classification of Diseases and Related Health Problems, 10th revision (ICD-10-CA) coding system for diagnoses. Because NACRS includes all A&E visits that take place in Ontario, for the purpose of this evaluation it is being used as a proxy for real-time A&E data streaming. Visits coded as ICD-10 Chapter 10 (Diseases of the Respiratory System) between April 2002 and March 2006 were used.

FluWatch, our gold standard, is Canada's Federal influenza surveillance system, which relies on the provincial legal requirement to report all laboratory-confirmed cases of influenza A and B, RSV and PIV to their provincial authority who, in turn, must report these numbers weekly to the Public Health Agency of Canada. These numbers are compiled weekly, are published under FluWatch, and are available within one week of receipt by FluWatch.

**Methods:** Weekly counts for each data source were visually compared against each other and Spearman rank correlation coefficients used to determine correlation between FluWatch (the gold standard) and Telehealth and NACRS. Time lag and relationships between multiple datasets at all possible time lags, were examined to ascertain whether Telehealth and/or NACRS were more timely than routine influenza surveillance. This method has been used with success within the context of syndromic surveillance, as it assumes that data are non-parametric, but makes no assumptions about the frequency distribution of variables, and does not make any assumption on the linearity between variables.

**Results:** These descriptive results' correlation coefficients suggest that the combined use of Telehealth and A&E data is successful at predicting influenza outbreaks in a timely manner. Further analyses will include testing the best combination of algorithms from the Telehealth data, or respiratory ICD codes, identifying the optimal method(s) of aberration detection, and comparing these results to those of other similar surveillance systems.

**Conclusion:** An additional level of preparedness for pandemic influenza in Ontario, Canada is potentially available using syndromic surveillance based on routine health service contact data.

5-031



### A European surveillance network for influenza antiviral resistance (VIRGIL)

**Lackenby, A.<sup>1</sup>; Lakhman, D.<sup>1</sup>; Sadler, C.<sup>1</sup>; Bedi, K.<sup>1</sup>; Sebastianpillai, P.<sup>1</sup>; Baldevarona, J.<sup>1</sup>; Thompson, C.<sup>1</sup>; Bennet, M.<sup>2</sup>; Gregory, V.<sup>2</sup>; Whittaker, L.<sup>2</sup>; Hay, A.<sup>2</sup>; Zambon, M.<sup>1</sup>**

<sup>1</sup>Influenza Laboratory, Virus Reference Division, Centre for Infection, HPA Colindale, UK; <sup>2</sup>National Institute for Medical Research, Department of Virology, UK

The global emergence of high levels of oseltamivir resistant influenza H1N1 virus occurred in the 2007-8 influenza season. This influenza virus strain was first detected in Europe through the activities of the European surveillance network for vigilance against viral resistance (VIRGIL). The VIRGIL program, funded by the EC through the Sixth Framework Research Programme (contract 503359) has been developed in collaboration with the European Influenza Surveillance Scheme (EISS) and constituent National Influenza Centres since 2004, as a European-wide surveillance network monitoring influenza virus susceptibility to both neuraminidase inhibitors (NIs) and M2 channel blockers. A representative subset of influenza virus strains which underwent antigenic characterisation in winter seasons 04/05 (N=231), 05/06 (N=415), 06/07 (N=270) and 07/08 (N=1067) from 28 countries in Europe were evaluated for susceptibility to NI drugs (oseltamivir and zanamivir) using a fluorescence IC<sub>50</sub> phenotype assay and to M2 channel inhibitors using pyrosequencing. During this period of time, there has been circulation of influenza B, H3N2 and H1N1 strains. Prior to 2007/8, less than 1% of strains showed alteration of susceptibility to NI drugs indicating that over this period of time there was no measurable increase in virus isolates with reduced susceptibility to NAI drugs in any subtype. The 2007-8 season was dominated by the influenza H1N1, and overall 20% of circulating H1N1 viruses in Europe were resistant to oseltamivir, caused by the presence of an H274Y mutation in the N1 neuraminidase protein. Levels of resistant virus varied between countries, with Norway having the highest incidence at 68% over the season, UK at 10%, but limited circulation in other European countries (1% in Italy). Resistant virus has emerged and circulated in countries with little or no drug use. The strains with 274Y remain sensitive to zanamivir and amantadine. Although 1-2% of influenza H1N1 strains from 2007-08 are amantadine resistant, no viruses with dual NI and amantadine resistance have been found. In contrast, the presence of amantadine resistance in H3N2 viruses has been particularly marked in recent influenza seasons. In the 05/06 season, the majority of circulating A/Wisconsin/67/2005-like H3N2 viruses (Clade 1 HA) in Europe possessed the S31N amantadine resistance mutation. However, viruses from the 06/07 winter season genetically more related to A/California/7/2004-like (Clade 2 HA) viruses possessed

an amantadine sensitive M2 gene indicating that amantadine resistance is not fixed amongst human H3N2 viruses and is virus clade dependent. This suggests that there is co-segregation of amantadine resistance with mutations elsewhere in the genome and that maintenance of amantadine resistance in circulating human H3N2 is unrelated to drug pressure. Relatively little H3N2 circulation was seen in 2007-8, but all of those available for testing were resistant to amantadine. The appearance of oseltamivir resistant and amantadine resistant virus demonstrates the need for continued extensive surveillance for antiviral resistance. The coordinated effort achieved by the VIRGIL network allowed global early warning of the emergence of significant antiviral resistance, and provided a central laboratory facility for those laboratories in European countries currently unable to undertake antiviral susceptibility testing.

5-032



### Multiplex RT-PCR for typing and subtyping of human influenza virus during a surveillance study

**Bharaj, P.; Broor, S.; Dhakad, S.; Singh, S.; Jain, P.; Kabra, S.K.**

AIIMS, India

**Introduction:** Influenza virus identification and detection is done by virus isolation (VI) in the MDCK cell line followed by hemagglutination assay (HA). Subtyping is done by hemagglutination inhibition (HI) assay. Limited reports are available on the application of reverse transcription-PCR (RT-PCR) for detection and typing of influenza viruses from clinical samples. During an ongoing community and hospital based surveillance, we decided to evaluate the utility of RT-PCR for detection and subtyping of human influenza viruses on clinical samples. Using published primers by Poddar S. in 2002, we applied a multiplex RT-PCR for the detection, typing and subtyping of influenza viruses in throat/nasal swabs and its comparison with virus isolation.

**Method:** published primers for detection and typing were used in the study. Matrix gene primers for detection of influenza A & B viruses were put in one tube. For subtyping influenza A to H3N2 and H1N1, HA/NA primers for all four subtypes were put in another tube. Multiplex PCR was standardized on standard strains of influenza A (H3N2 and H1N1) and influenza B viruses. RNA extraction from clinical samples was done using an RNeasy kit (Qiagen). cDNA was prepared using AMV RT enzyme and random hexamer primers. Standardized RT-PCR was applied on 207 throat swabs collected during the surveillance study. Results: Two thousand one hundred throat swabs were collected

from December 2004-February 2008. Two hundred and seven samples were randomly selected for evaluation of RT-PCR over VI for detecting the presence of influenza virus RNA. Samples were selected from July 2007-December 2007 wherein maximum detections of influenza virus were achieved by a traditional method. VI followed by HI in these 207 samples revealed the presence of 19 influenza A (1 H3N2 and 18 H1N1) and 14 influenza B isolates. RT-PCR detected the presence of influenza A in 21 (1 H3N2 and 20 H1N1) and 16 influenza B samples (proposal: detected the presence in 21 influenza A (1 H3N2 and 20 H1N1) and influenza B in 16 samples. Hence, RT-PCR detected the presence of influenza virus RNA in 4 (1.9%) additional samples. There was perfect correlation (100%) between types and subtypes obtained by VI and RT-PCR when samples were positive by both methods.

**Conclusions:** Multiplex RT-PCR can be successfully used in surveillance, providing excellent correlation with traditional methods of influenza virus identification and allowing sensitive, rapid detection.



5-033

### Influenza in a prospective community-based pediatric cohort in Nicaragua

**Gordon, A.<sup>1</sup>; Saborio, S.<sup>2</sup>; Kuan, G.<sup>3</sup>; Vide, E.<sup>4</sup>; Ortega, O.<sup>4</sup>; Reyes, M.<sup>3</sup>; Reingold, A.<sup>1</sup>; Balmaseda, A.<sup>2</sup>; Harris, E.<sup>5</sup>**

<sup>1</sup>University of California, Berkeley, USA; <sup>2</sup>National Center for Diagnosis and Reference, Ministry of Health, Nicaragua; <sup>3</sup>Socrates Flores Vivas Health Center, Ministry of Health, Nicaragua; <sup>4</sup>Sustainable Sciences Institute, Nicaragua; <sup>5</sup>School of Public Health, University of California, Berkeley, USA

**Background:** Major questions still remain surrounding the epidemiology of influenza in tropical countries, such as seasonality of virus activity and disease burden. Here we characterize the epidemiology of influenza in a prospective cohort of Nicaraguan children.

**Methods:** In June 2007, we began a prospective community-based cohort study, embedded within an ongoing dengue cohort study of children 2-12 years of age, to examine the incidence of laboratory-confirmed influenza and analyze risk factors associated with influenza virus infection in Managua, Nicaragua. Nasal and throat swabs were collected from a random 20% of all cohort participants that presented for a medical appointment with influenza-like illness, defined as a combination of fever or a history of fever with cough and/or sore throat of 4 days or less duration. Samples were tested for influenza viruses by RT-PCR, and viral isolation was performed on all RT-PCR-positive

samples. Virus isolates are currently undergoing full-length sequencing. Additionally, all samples were tested for Respiratory Syncytial Virus (RSV), Parainfluenza 1-3, Adenovirus, and Human Metapneumovirus (HMPV) to investigate the relative contribution of these viruses to respiratory illnesses in Nicaraguan children. Weekly influenza incidence in the cohort was estimated by applying the percentage of samples positive for influenza in the calendar week to the total number of children that presented with influenza-like illness, divided by the person-time for that week.

**Results:** A total of 3,285 children between the ages of 2 and 12 years old were followed for every primary care appointment between June 2007 and May 2008. To date, 680 swab samples have been collected and tested for influenza virus A and B, of which 80 (12%) were positive by RT-PCR. The estimated incidence of influenza in the cohort during this first year of the study was 21.1 cases per 100 person-years. A peak of influenza A/H2N3 was seen in June of 2007, with the highest weekly estimate of influenza burden reaching 221 cases per 100 person-years. Influenza A and B virus activity was also elevated from January through March of 2008.

**Conclusions:** These initial results from the first year of the study indicate that influenza is a major health concern among Nicaraguan children and, together with data from previous years, suggest that Nicaragua experiences a peak of influenza activity between May and July of each year and may experience a second peak of influenza some years between November and January. This is the first large-scale prospective study to provide data on the incidence and seasonality of influenza and other respiratory viral diseases in Central America.



5-034

### Influenza sentinel surveillance in Georgia

**Machabishvili, Ann<sup>1</sup>; Hay Alan, H.A.<sup>2</sup>; Imnadze Paata, I.P.<sup>1</sup>**

<sup>1</sup>National Center for Disease Control and Public Health, Georgia; <sup>2</sup>WHO Influenza Centre, National Institute for Medical Research, UK

The first and last case of avian influenza A/H5 in Georgia was detected by real time RT-PCR in wild swans from the Black Sea region of the country in 2006.

The emergence of an influenza virus of pandemic potential (A/H5N1) and absence of data on influenza strains circulating in Georgia have vividly shown the importance of sensitive surveillance of Influenza-Like Illnesses (ILI) in the country and therefore an influenza laboratory was established at the National Center for Disease Control and Public Health in 2006. Isolation of influenza viruses on MDCK cells and hemagglutination inhibition

were used for influenza virus detection and subtyping; further antigenic study and gene sequencing were performed at the WHO Influenza Centre, London. In 2006, a total of 34 influenza viruses were isolated: 26 A/H3N2 and 8 influenza B; and in 2007 a total of 27 isolates – 18 A/H3N2, 7 A/H1N1 and 2 influenza B. Isolation of influenza viruses was mostly during January and March when the peak incidence of influenza and ILI occurred. The H3N2 viruses were antigenically closely related to A/Wisconsin/67/2005-like reference viruses. The HA sequences obtained for some of the 2006 isolates were close to that of A/Wisconsin/67/2005, whereas those for 2007 isolates fell within the phylogenetic subgroups represented by A/Nepal/921/2006 or A/Brisbane/10/2007. The H1N1 viruses were all antigenically more closely related to A/New Caledonia/20/99-like reference viruses<sup>1</sup>, although the HA sequences of some fell within a subclade of the A/Solomon Islands/3/2006 clade which lacks the K140E change characteristic of A/Solomon Islands/3/2006-like viruses. The B viruses isolated during both seasons were of the B/Victoria-lineage and their HAs were antigenically and genetically closely related to the vaccine virus B/Malaysia/2506/2004. Antigenic and genetic analyses revealed that the strains isolated in Georgia were similar to those circulating worldwide.

5-035

# **Exposure to contagious health care workers and patients increases the risk of transmission of hospital-acquired influenza-like illness for patients**

**Voirin, N.<sup>1</sup>; Roche, S.<sup>2</sup>; Barret, B.<sup>3</sup>; Ecochard, R.<sup>4</sup>; Vanhems, P.<sup>1</sup>**

<sup>1</sup>Université de Lyon; Université Lyon<sup>1</sup>; CNRS; UMR 5558; Hospices Civils de Lyon, Hôpital Edouard Herriot, France; <sup>2</sup>Hospices Civils de Lyon, Service de Biostatistique; Université de Lyon; Université Lyon<sup>1</sup>; CNRS; UMR 5558, France; <sup>3</sup>Sanofi Pasteur, Affaires médicales globales, Département d'épidémiologie, France; <sup>4</sup>Hospices Civils de Lyon, Service de Biostatistique; Université de Lyon; Université Lyon<sup>1</sup>; CNRS; UMR 5558, Biostatistique Santé, France

**Introduction:** Nosocomial outbreaks of influenza-like illness (ILI) are frequent and all types of hospital wards can be affected with a patient attack rate ranging from 11% to 59% and mortality rate up to 66%.

During their hospital stay, patients may acquire ILI from infectious health care workers (HCWs), patients or visitors, but there is no precise quantification of this risk.

The objective was to estimate the risk for patients of acquiring nosocomial ILI during their hospital stay and to compare that risk to that of community acquired ILI.

**Methods:** A prospective study has been ongoing since November 2004 at the Edouard Herriot University Hospital, Lyon, Rhone-Alpes region, France. The analysis presented here comes from the data of the first season of influenza surveillance conducted from November 15, 2004 to April 15, 2005 in 12 hospital units.

Units were surveyed daily to detect cases of ILI among patients or HCWs. ILI was defined as fever  $\geq 37.8^{\circ}\text{C}$  associated with cough or sore throat. A case was considered nosocomial if onset of ILI occurred the day of admission or later. Non-cases were patients or HCWs free from ILI during the study period. Hospital data (admission date, clinical information, etc) on cases and non-cases were extracted from the hospital information system (PMSI).

In each unit, exposure of each patient to other patients and to HCWs was monitored on a half-day basis. Exposure was defined as the presence in the same hospital unit during the same half-day of at least two individuals. An exposure of a patient to a contagious HCWs or patient within the 10 half-days preceding ILI onset was assumed compatible with a transmission. Individuals were considered contagious from 1 day preceding their onset of ILI to 5 days afterwards. Patients with ILI onset before admission were not counted as at risk of nosocomial ILI but were considered contagious for other patients.

The incidence rate of nosocomial ILI cases was compared with the incidence rate in the community using age-standardized incidence ratios (SIRs). Standardization on age allowed for differences in age structure between hospital and community populations. Data on ILI incidence in the Rhone-Alpes region (6,000,000 inhabitants) came from the French sentinel surveillance network. These were estimations by five-year age classes of the weekly number of new ILI cases seen by all general practitioners. Using the surveillance network data, the absolute risk of ILI for each patient during each half-day was calculated assuming that the risk at hospital was equal to the risk within the community. The sum of this risk over the whole study period gave the expected number of ILI.

The SIR of ILI over the whole study period was computed as the ratio of total number of observed to total number of expected ILI. SIR variations were also studied taking into account the immune status of the patients and the source of contamination (HCW, patient or both). Immunosuppression was defined on the basis of the International Classification of Diseases version 10. SIRs were considered statistically significant if their 95% confidence interval (CI) did not include 1.

**Results:** A total of 24 nosocomial ILI cases were observed during 4059 patient-weeks of stay. The expected number of cases during the same period was 18. This led to an overall SIR of 1.33 (95%CI 0.85-1.98).

A significant heterogeneity of the SIRs was observed between the 12 units ( $p < 0.00001$ ). The SIR for immunosuppressed and non-immunosuppressed patients was 2.35 (95%CI 1.21-4.11) and 0.93 (95%CI 0.48-1.62) respectively.

Patients with no documented exposure to infectious HCWs or

patients, had a SIR of 0.66 (95% CI 0.32-1.22), while patients with established exposure to at least one infectious HCW or patient, had a SIR of 4.73 (95%CI 2.58-7.94).

Among patients with established exposure, patients exposed to at least one contagious HCW but not to a contagious patient had a SIR of 3.13 (95%CI 0.35-11.28), patients exposed to at least one contagious patient but no contagious HCW had a SIR of 4.43 (95%CI 1.78-9.13) and patients exposed to at least one contagious patient and one contagious HCW had a SIR of 6.76 (95% CI 2.18-15.77).

**Conclusion:** The risk for patients of acquiring ILI within hospital saw a significant 4-fold increase in cases of exposure to contagious health care workers or patients.

To our knowledge, this is the first study providing an estimation of the risk of hospital acquired ILI among patients with individual-based measurement of exposure to contagious HCWs or patients.

The results confirmed that both HCWs-to-patient and patient-to-patient transmission routes are significant. Preventing HCWs-to-patient transmission may depend on vaccination of HCWs as well as on standard hygiene procedure. However, control measures needs to be further explored in a larger setting.

These preliminary results may be particularly interesting in the case of influenza pandemic and will be completed using the same approach by the data from the 2005/2006 and 2006/2007 seasons carried out at the Edouard Herriot Hospital in 33 and 36 units respectively.

## 6 ANTIVIRALS AND RESISTANCE

6-002

### Guidelines for the treatment and prevention of seasonal influenza: a comparative review of antiviral recommendations in the European Union

*Stephenson, Iain; Clarke, T.W.; Pareek, M.*

*University Hospitals Leicester, UK*

**Objective:** To evaluate differences among recommendations for the use of antiviral drugs in the treatment and prevention of seasonal influenza across Europe.

**Methods:** Guidelines issued between 1 Jan 2003 and 1 Sept 2007 were retrieved by web searches, and where required translated into English. Parameters considered important for management of influenza were evaluated. Guidelines were scored using the AGREE appraisal instrument.

**Results:** Guidelines were obtained from 8 European countries: France, Germany, Italy, Netherlands, Poland, Portugal, Sweden and the United Kingdom. Most guidelines preferentially recommend neuraminidase inhibitors, but 3 countries were unclear in recommendations or suggested M2 inhibitor use in some circumstances. Clinical diagnosis of eligible patients during periods of influenza activity is acceptable except in Poland where virological confirmation is required before treatment. Guidelines generally recommend antiviral use in patients at high risk of complications of influenza, except in Germany where there is a strong recommendation to treat all patients presenting with influenza. Post-exposure prophylaxis for household contacts is recommended in Sweden and Germany, but not in other countries. Only UK guidelines have been updated since first publication. All scored fairly poorly by the AGREE instrument although French, Polish, Swedish and UK guidelines were recommended.

**Conclusion:** There are major variations in the recommendations for treatment and prevention of seasonal influenza. The development of pan-European guidance for management of seasonal influenza should be considered. Updating is important for reflecting emerging patterns of antiviral resistance. A central repository to hold guidelines would improve the accessibility of clinical practice recommendations

6-003

### HydrolEasy assay for rapid detection of oseltamivir resistance in H1N1

Nielsen, Lars<sup>1</sup>; Nielsen, C.<sup>2</sup>; Bragstad, K.<sup>1</sup>; Christensen, U.<sup>2</sup>

<sup>1</sup>National Influenza Laboratory, Denmark; <sup>2</sup>PentaBase, Soendersoe, Denmark

**Background:** An increasing number of H1N1 viruses with the H274Y mutation associated with the oseltamivir resistant phenotype was observed in many countries during the 2007/2008 influenza season. The resistant virus is fully able to cause human-to-human infection and there is no indication of a reduced virulence. For clinical purposes, a rapid assay testing such a resistant mutation is needed.

**Methods:** We developed an assay based on specific and sensitive HydrolEasy® probe technology (PentaBase). HydrolEasy® probes can be made shorter and more specific than their DNA counterparts and increase the signal-to-noise ratio. We developed a dual-plex assay, having one probe that detects the presence of wild-type H1N1 (green channel) and another that specifically detects the presence of the H274Y mutation (yellow channel). This assay was tested on both a Ro-torgene® 6000 (Corbett) and a Mx3005p (Stratagene) using a modified TaqMan® protocol. The results were compared to DNA sequencing of the PCR products.

**Results:** The assay was able to detect resistant mutations in all strains that were detected by sequencing.

**Conclusion:** We have developed an assay for detection of oseltamivir resistance that can be performed in a few hours. Such an assay may have a clinical impact when treating patients with antiviral drugs.

6-004

### Susceptibility to neuraminidase inhibitors of influenza viruses circulating in Spain

Ruiz, Guillermo<sup>1</sup>; Correia, V.<sup>2</sup>; Lackenby, A.<sup>3</sup>; Casas, I.<sup>1</sup>; Rebelo de Andrade, H.<sup>2</sup>; Zambon, M.<sup>3</sup>; Pérez-Breña, P.<sup>1</sup>

<sup>1</sup>Centro Nacional de Microbiología, Instituto de Salud Carlos III, Spain; <sup>2</sup>National Influenza Centre, Respiratory and Enterovirus Unit, National Institute of Health, Dr. Ricardo Jorge, Portugal; <sup>3</sup>Health Protection Agency, Centre for Infection, UK

**Introduction:** After the introduction of neuraminidase inhibitors (NAI) into clinical practice in 1999, several reports of increasing frequency of oseltamivir resistance in treated children and immunocompromised patients indicate the appearance and transmission of resistant strains. Furthermore, during the 2007/08 influenza season several countries from Europe detected high

rates of H1N1 influenza viruses resistant to oseltamivir. This setting led us to conduct a study of the antiviral susceptibility to NAI in selected influenza viruses isolated in different Spanish regions from 2004 to 2008.

**Methods:** The genotypic susceptibility to NAI was assessed by NA gene sequence analysis in 50 A(H3N2) and 33 A(H1N1) influenza viruses. The oseltamivir susceptibility was also determined in 12 A(H3N2) viruses from the 2004/05 season, 5 A(H3N2) from 2006/07 and 18 B from 2005/06 using a fluorescence based neuraminidase inhibition assay with MUNANA (2'-(4-Methylumbelliferyl)- $\alpha$ -D-N-acetylneuraminic acid) as the fluorogenic substrate. The results were expressed as 50% inhibitory concentration (IC<sub>50</sub>). For comparison and confirmatory purposes, the outliers were tested with the chemiluminescence based neuraminidase inhibition assay with the NA-Star® commercial kit.

**Results:** The sequencing of the NA gene revealed an E119V substitution in three consecutive influenza A(H3N2) isolates taken from an immunocompromised patient in the 2004/05 season. These resistant viruses also had the V27A mutation related to adamantane resistance. One H274Y mutation was found in an influenza A(H1N1) virus isolated during 2007/08.

The phenotypic assays showed that the median of IC<sub>50</sub> for oseltamivir in influenza B viruses was 18.37 nM and 4 minor outliers were identified: 30.22, 31.43, 31.90 and 34.81 nM. The minor and major cut-off values were 28.91 and 41.92 nM, respectively. For the A(H3N2) influenza viruses the median of IC<sub>50</sub> was 0.24 nM with minor and major cut-off values of 0.74 and 1.85 nM in that order. The 3 viruses with the E119V mutation in the NA segment showed IC<sub>50</sub> values of 29.27, 9.97 and 17.85 nM. The sensitive A(H3N2) viruses determined by NA sequencing did not reveal phenotypic resistance by fluorescence based assay.

In the chemiluminescence assay, the minor outlier for influenza B with the highest value in the fluorescence assay (34.81 nM) and the 3 resistant A(H3N2) influenza viruses showed lower IC<sub>50</sub> values: 2.72 nM for the influenza B virus and 3.97, 2.79 and 4.35 nM for the resistant A(H3N2) viruses. Nevertheless, the resistant A(H3N2) viruses exhibited >20-fold increase in IC<sub>50</sub> in relation to the sensitive control A/Wisconsin/67/05 (IC<sub>50</sub>: 0.16 nM).

**Conclusions:** I. The genotypic results were in agreement with the phenotypic fluorescence based method. II. In spite of the lower values of IC<sub>50</sub> achieved with the NA-Star® kit, both assays were able to detect A(H3N2) resistant viruses with the E119V mutation. III. The median and cut off values obtained with fluorescence based assay for influenza A and B viruses were similar to the values reported in Europe for the seasons considered. IV. Only one resistant virus showing the H274Y mutation has been found in Spain during the 2007/08 season, despite the fact of being surrounded by countries with medium to high rates of A(H1N1) resistant viruses.

6-005

### Oseltamivir-sales in Norway prior to the emergence of oseltamivir resistant influenza A(H1N1) virus in the 2007/2008 season

**Hauge, Siri Helene;** Borgen, K.; Blix, H.S.; Hungnes, O.; Dudman, S.; Aavitsland, P.

Norwegian Institute of Public Health, Norway

**Background:** On January 25, 2008, we notified the WHO under International Health Regulations of an unusually high proportion (12 of 16) of oseltamivir resistant influenza A(H1N1) strains among the samples collected as part of the seasonal routine surveillance. Sequence analysis of the viral neuraminidase gene showed that the strains carried a mutation resulting in a substitution of histidine by tyrosine at amino acid position 274. During the whole season, the proportion of oseltamivir resistant viruses was almost 70%. In order to see if the emergence of oseltamivir resistant influenza A(H1N1) viruses in the 2007/2008 season was preceded by a high sale of oseltamivir, we assessed the sale of this drug for the years 2004-2007.

**Methods:** We collected sales figures for oseltamivir from two nationwide Norwegian databases for the years 2004-2007; the wholesaler database covering total sales of oseltamivir to pharmacies and institutions, and the Norwegian Prescription Database (NorPD) covering all prescriptions filled by outpatients in Norway. Oseltamivir is not available over the counter in Norway. Our outcome measures were number of oseltamivir courses sold and number of oseltamivir prescriptions filled. Using population statistics combined with sales figures from the wholesale database and the Norwegian Prescription Database, we calculated prescriptions filled and courses sold per 1000 inhabitants by year.

**Results:** The number of oseltamivir courses sold from wholesalers was in 2004 699 (0.15 per 1000), in 2005 66249 (14.38 per 1000), in 2006 33573 (7.24 per 1000) and in 2007 4686 (1.00 per 1000). Data from the Norwegian Prescription Database showed that in 2004, 778 individuals (0.17 per 1000) filled a prescription for oseltamivir in Norway. In 2005, the figure was 23308 (5.06 per 1000), in 2006 4826 (1.04 per 1000) and in 2007 3464 (0.74 per 1000 (preliminary data, final data will be presented)).

**Conclusions:** Oseltamivir is rarely sold in Norway. The higher sales figures for 2005 were probably caused by families stockpiling the drug in fear of a pandemic. Still, oseltamivir resistant influenza A(H1N1) virus has spread in the Norwegian community in the 2007/2008 season. This independence from an antiviral selection pressure means that the H274Y mutant influenza A(H1N1) viruses may not have lower fitness than wild-type viruses.

6-006

### Fatal influenza due to oseltamivir resistant H1N1 with full characterization using whole viral genome sequencing

**Bragstadt, Karoline<sup>1</sup>;** Astrup, B.S.<sup>2</sup>; Jensen, T.G.<sup>3</sup>; Nielsen, L.P<sup>1</sup>

<sup>1</sup>National influenza Laboratory, Denmark; <sup>2</sup>Department of Forensic Medicine, University of Southern Denmark, Denmark; <sup>3</sup>Department of Clinical Microbiology, Odense University Hospital, Denmark

In the influenza season 2007/08 the Northern Hemisphere and especially Europe detected strains of influenza A H1N1 with resistance towards oseltamivir. Usually H1N1 causes less severe infections than H3N2 and it was claimed that resistant virus has lower replication rates and thus may have decreased virulence. We report a fatal case of influenza due to H1N1 influenza A virus complicated with pneumonia with *Staphylococcus aureus* producing tsst-1. The H1N1 influenza A virus was resistant to oseltamivir and we report the full characterization of the viral genome.

**Methods:** A fatal case of pneumonia in a previously healthy 8 year-old boy, who was sent to the Institute of Forensic Medicine, Odense University Hospital. Influenza A was detected by real-time RT-PCR and confirmed to be of the H1N1 subtype. The virus could be demonstrated in the sputum as well as in the lung tissue. The virus was subjected to whole genome sequencing for identification of amino acid differences between the lethal case virus and other oseltamivir resistant and non-resistant viruses.

**Results:** The sequence of the neuraminidase gene showed the H274Y substitution, which indicates resistance towards oseltamivir, but lacked the I38L substitution present in many recent resistant viruses. Interestingly, the lethal case virus possessed a PB1 R260K substitution not found in other Danish resistant viruses. (The resistant viruses differed from the non-resistant Danish viruses with substitutions in the NA, PB2, PB1 and in the NP proteins.) No mutation known to be associated with increased virulence was found.

**Conclusion:** H1N1 with the oseltamivir resistance H274Y mutation in the NA gene can cause fatal influenza infections.

## POSTER PRESENTATIONS

6-007

### Expression of recombinant human interferon IFN- $\lambda$ 1, $\lambda$ 2 and $\lambda$ 3 in A549 cells and their antiviral activity in replication of influenza virus

**Betakova, Tatiana<sup>1</sup>**; Svetlikova, D.<sup>1</sup>; Ohradanova, A.<sup>1</sup>; Kabat, P.<sup>2</sup>

<sup>1</sup>Institute of Virology, Slovakia; <sup>2</sup>Comenius University, Bratislava, Slovakia

Recently discovered type III interferons (IFN-lambda 1, 2 and 3) are a novel and non-typical representative of the human IFN family. Despite the fact that IFNs have been defined at the gene and protein levels, the full spectrum of their antiviral activities has not been sufficiently characterized. Such a "white spot" in knowledge of IFN-lambda biology represents a target for our work. Expression of IFN-  $\lambda$ 1, IFN-  $\lambda$ 2 and IFN-  $\lambda$ 3 was induced by poly(I)\*poly(C) and total RNA was extracted. Isolated RNA was reverse transcribed into cDNA. The gene specific primers were used to amplify interferons from cDNA. PCR products were cloned into the pCR2.1 vector. The correct sequence and orientation have been verified by sequencing. PCR products were then recloned into pcDNA3.1(+) and transfected into the A549 cells. A549 cells expressing lambda interferons were infected with the influenza virus (H7N1). The ability of IFNs to inhibit replication of influenza virus in A549 cells was evaluated.

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6-008

### Peptide-based entry inhibitors for influenza

**Voss, Thomas<sup>1</sup>**; LeBlanc, C.S.<sup>1</sup>; Kulakosky, P.<sup>2</sup>; Wilson, R.<sup>2</sup>; Garry, R.F.<sup>1</sup>

<sup>1</sup>Tulane University School of Medicine, USA; <sup>2</sup>Autoimmune Technologies, LLC, USA

Influenza infections are responsible for seasonal epidemics and less frequent pandemics responsible for millions of infections and thousands of deaths annually worldwide. Currently available influenza therapeutics target later stages of virus replication and while they are effective in many cases, there is increased resistance to these inhibitors in clinical settings and there is little evidence that they are effective in reducing transmission of virus to susceptible individuals. We have developed a peptide-based therapeutic platform targeting viral envelope glycoproteins for a number of human viruses including influenza viruses. In vitro studies show the prototype peptide to be effective at inhibiting a wide variety of influenza A and influenza B viruses

at nM concentrations using a plaque inhibition assay. In vivo studies using the ferret model show robust antiviral activity in therapeutic or post-exposure prophylaxis regimens treating by intranasal administration. In addition, the lead therapeutic peptide candidate was shown to be active in reducing transmission when infected ferrets were treated and co-housed with naïve untreated cage mates and when treated naïve ferrets were co-housed with infected cage mates. Taken together, these data support the advanced development of entry inhibitors for seasonal and pandemic influenza, adding a broad-spectrum therapeutic to currently approved influenza therapeutics.

6-009

### Effects of antivirals on severity and duration of symptoms, viral shedding, and household transmission of influenza

Cowling, B.J.<sup>1</sup>; **Cheng, K.Y.<sup>1</sup>**; Fang, V.J.<sup>1</sup>; Chan, K.H.<sup>2</sup>; Uyeki, T.M.<sup>3</sup>; Houck, P.M.<sup>4</sup>; Peiris, J.S.M.<sup>2</sup>; Leung, G.M.<sup>1</sup>

<sup>1</sup>Department of Community Medicine and School of Public Health, the University of Hong Kong, China; <sup>2</sup>Department of Microbiology, the University of Hong Kong, China; <sup>3</sup>Influenza Branch, National Center for Infectious Diseases, CDC, China; <sup>4</sup>Division of Global Migration and Quarantine, National Center for Infectious Diseases, CDC, China

**Background:** Several large controlled trials have demonstrated that antivirals can reduce the duration and severity of illness if taken sufficiently early after symptoms begin. However there are few estimates of the degree to which antivirals reduce the transmissibility of influenza. We assessed the effect of antiviral use on clinical disease and infectivity as part of a large community study of influenza transmission in households in Hong Kong.

**Methods:** Subjects older than 2 years with confirmed influenza infection by QuickVue Influenza A+B test were recruited from 30 outpatient clinics in Hong Kong during the 2007 and 2008 influenza seasons. Nose and throat swabs were collected from all household members at an initial home visit within 36 hours of recruitment and 3 and 6 days later, to determine influenza infection by viral culture and quantitative RT-PCR. Clinical influenza in secondary contacts was assessed by self-reported symptom diaries.

**Results:** A total of 128 households were recruited in 2007 and in the current year a further 400 households will have been recruited by August 31, 2008. Based on data from the first 128 households, index cases that were prescribed antivirals were less likely to infect their household contacts (adjusted OR 0.69, 95% CI: 0.13, 3.54). Those given oseltamivir had fewer symptoms (average number of symptoms = 2.03 vs 2.43), which were also resolved 0.4 days sooner. In contrast, index cases prescribed amantadine showed little reduction in symptom duration or severity. The

average times from recruitment to undetectable viral load for antiviral treatment were found to be shorter for oseltamivir (0.92 days) compared to amantadine (1.42 days) or neither (1.61 days). Results based on the final dataset including virologic outcomes will be available by the date of the conference.

**Discussion:** Our interim findings suggest that oseltamivir can reduce the severity and duration of influenza-like illness symptoms, similar to other previous studies, whereas amantadine has no observable effect on symptom severity or duration, although we did not determine resistance profile. Oseltamivir use may also reduce secondary infections within households.

6-010

### Oseltamivir resistant influenza A(H1N1) viruses and baseline patient clinical characteristics during the 2007/2008 influenza season in Europe

**W. John Paget**<sup>1</sup>, Tamara J. Meerhoff<sup>1</sup>, , Olav Hungnes<sup>2</sup>, Sylvie van der Werf<sup>3</sup>, Therese Popow-Kraupp<sup>4</sup>, Adam Meijer<sup>1,5</sup>, Koos van der Velden<sup>6</sup>, Alan Hay, Maria Zambon<sup>8</sup> on behalf of EISS\*.

<sup>1</sup> European Influenza Surveillance Scheme Co-ordination Centre, NIVEL, Utrecht, the Netherlands, <sup>2</sup> Norwegian Institute of Public Health, Nydalen, Norway, <sup>3</sup> Pasteur Institute, Paris, France, <sup>4</sup> Institute of Virology, Vienna, Austria, <sup>5</sup> National Institute for Public Health and the Environment, Bilthoven, the Netherlands, <sup>6</sup> Radboud University Medical Centre, Nijmegen, the Netherlands, <sup>7</sup> WHO Collaborating Centre for Reference and Research on Influenza, Mill Hill, London, United Kingdom, <sup>8</sup> Health Protection Agency, London, United Kingdom.

\* Substantial data was collected for Germany. Persons involved were: Brunhilde Schweiger, Susanne Duwe, Stefan Brockmann, Silke Buda and Udo Buchholz.

**Background:** Antiviral resistance of influenza viruses in Europe is being monitored via the European surveillance network for vigilance against viral resistance (VIRGIL) and the European Influenza Surveillance Scheme (EISS). Although the use of antivirals is very low in Europe, a substantial increase in oseltamivir resistant A(H1N1) viruses was reported during the 2007-2008 influenza season. Our objective is to describe baseline demographic and clinical characteristics of patients with oseltamivir resistant (Os-res) viruses and oseltamivir susceptible (Os-sus) A(H1N1) viruses, and to identify patient-related risk factors for oseltamivir resistance.

**Methods:** Through the EISS-VIRGIL antiviral database, data is collected on influenza virus (sub)type, number of A(H1N1) viruses tested for resistance, number of A(H1N1) Os-res viruses, geographic location, age, gender, date of onset disease, date specimen collected, exposure to antivirals of patient and/or household contact, complications, hospitalisations and deaths. Data from week 40/2007 to week 20/2008 will be included in

the final analysis. Results will be provided for: Os-res and Os-sus viruses, and on significant factors associated with oseltamivir resistance.

**Results:** By mid-May, the database contained information on 2727 A(H1N1) virus isolates tested for resistance, of which 24.5% were resistant to oseltamivir. To date, clinical patient characteristics were available for 1184 isolates (43% of total database), with 23.2% resistance coming from 10 countries, and the data therefore being preliminary. Oseltamivir resistance occurred in all age groups. There were no significant differences in age distribution and gender between patients with Os-res viruses and patients with Os-sus viruses. To date, one person reported the use of antivirals and was not infected with Os-res virus. Out of six cases with household contacts exposed to antivirals, three were infected with Os-res virus and three with Os-sus susceptible virus. Potential association of individual factors with infection with Os-res viruses will be tested in a univariate analysis. Multivariate logistic regression analysis will be performed to compute estimated odds ratios adjusted for all other factors in the models.

**Conclusions:** A substantial proportion of oseltamivir resistant viruses was observed during the 2007-2008 influenza season in Europe. It is important to identify possible patient-related risk factors for oseltamivir resistance. These analyses are ongoing and their results will become available after the influenza season has come to an end.

6-011

### Targeting NS1 for drug discovery and identification of novel anti-influenza compounds

Basu, D.<sup>1</sup>; Auble, D.T.<sup>2</sup>; Engel, D.A.<sup>1</sup>

<sup>1</sup>Department of Microbiology, University of Virginia School of Medicine, USA;

<sup>2</sup>Department of Biochemistry and Molecular Genetics, University of Virginia School of Medicine, USA

A yeast-based system was developed for the identification of novel anti-influenza compounds that target the NS1 protein. A high-copy yeast plasmid with a GAL1 promoter-driven NS1 cDNA derived from A/WSN/33 was placed in an *S. cerevisiae* strain that carried null alleles for the drug efflux regulators PDR1 and PDR3. Growth in a galactose-containing medium resulted in expression of NS1 protein and a pronounced slow-growth phenotype. Identification of potential anti-NS1 compounds was carried out by screening the NCI Diversity Set library for compounds that could suppress the NS1-induced slow-growth phenotype. The test strain was grown overnight in media containing raffinose and then diluted in media containing raffinose plus galactose to induce NS1 expression. Cells were plated in 96 well plates in

the presence of 50  $\mu$ M drug or DMSO as control, and growth was monitored by OD over a two-day period. Hits producing a significant increase in growth relative to the DMSO-treated control cells were identified and tested again using independent stocks of each compound. A total of 5 reproducible hits from the approximately 2000-member Diversity Set library were identified. Several controls were carried out to characterize the initial hits. To rule out specific effects on transcription from the GAL1 promoter, or on general aspects of DNA metabolism, cells were constructed with the NS1 cDNA in a high-copy plasmid under control of the CUP1 promoter, or with the NS1 cDNA integrated into the yeast cell genome. Only compounds that showed activity in both systems were retained for further study. In addition, a parallel screen against the influenza M2 protein from A/WSN/33, which also produced a slow-growth phenotype in yeast, identified a non-overlapping set of hits compared to the anti-NS1 screen. Since the M2 expression construct was otherwise identical to that used for expression of NS1, this indicated that the anti-NS1 hits were not targeting functions that control expression of the foreign protein in yeast, or other aspects of plasmid DNA metabolism. Furthermore, western blot analysis indicated that the anti-NS1 hits had no effect on the level of NS1 expression during an 8-hour period of drug treatment. Hits were used to challenge virus replication of A/PR/8/34 and A/HongKong/19/68 in MDCK cells. They resulted in a 10 – 100 fold decrease in replication as measured by TCID<sub>50</sub> analysis over a concentration range of 10 – 50 micromolar. The compounds did not inhibit growth of MDCK cells during a period corresponding to the time course of viral infection and were non-toxic or minimally toxic to cells when incubated for up to 6 days. Consistent with inhibition of NS1 function, the compounds reversed the NS1-dependent inhibition of IFN-beta mRNA accumulation in virus infected cells and resulted in decreased accumulation of viral RNAs.

6-012

### Enhanced surveillance to monitor neuraminidase inhibitor resistance in the U.S. during the 2007-08 season

**Sheu, T.G.;** Okomo-Adiambo, M.; Deyde, V.M.; Wallis, T.R.; Hall, H.; Xu, X.; Dharan, N.; Fry, A.M.; Klimov, A.I.; Gubareva, L.V.

*Centers for Disease Control and Prevention, USA*

**Introduction:** Continuous evolution of influenza viruses necessitates the update of seasonal vaccine strains and requires the close monitoring of antigenic changes in the surface antigens, hemagglutinin and neuraminidase (NA). Vaccination provides the most effective means for control of influenza infections, although there is a definitive niche for antiviral use in the prophylaxis and treatment of influenza infections. Two classes of drugs, M2

blockers (adamantanes) and NA inhibitors (NAIs), are currently available for the control of influenza infections in several countries, including the U.S. Oral oseltamivir and inhaled zanamivir are FDA-approved NAIs that are active against both influenza A and B infections, whereas M2 blockers are not effective against influenza B viruses. Monitoring resistance to anti-influenza drugs became an integral component of strain surveillance conducted at the WHO Collaborating Centers, including the Influenza Division at CDC. In recent years, a dramatic increase of resistance to the M2 blockers among influenza A viruses has been detected worldwide. Since the 2005-06 influenza season, the use of NAIs instead of adamantanes has been recommended by CDC and public health agencies in other countries.

At CDC, adamantane resistance among influenza A viruses has been monitored using a high throughput pyrosequencing approach which allows detection of resistance markers in the M2 gene; whereas the chemiluminescent NA inhibition assay has been the primary screening tool for detection of resistance to NAIs. Although the NA inhibition assay is relatively rapid, it has several limitations, including a requirement for grown and (sub) typed viruses. Moreover, viruses that show reduced susceptibility to NAI need to be further analyzed genetically to identify known or novel markers of drug-resistance. The number of viruses tested per season has increased over time and the frequency of NAI-resistance in past influenza seasons has remained very low (<1%) among influenza A and B viruses circulating in the U.S. and other countries worldwide.

In the early 2007-08 influenza season, a small (5.5%), but noticeable, increase in resistance to oseltamivir was detected among influenza A(H1N1) viruses circulating in the U.S. Around the same time, a very high frequency of oseltamivir-resistance was reported for A(H1N1) viruses circulated in Norway (up to 66%) and some other countries in Europe. All oseltamivir-resistant A(H1N1) viruses shared the same mutation (H274Y) in the NA gene. These events prompted CDC to enhance surveillance efforts within the U.S. and to develop new approaches for drug resistance detection.

### Methods: Conventional Surveillance for Antiviral Resistance

According to previously established procedure, (sub)typed virus isolates submitted to the Influenza Division for antigenic and genetic characterization during the 2007-08 influenza season were screened for antiviral resistance after additional propagation at CDC. Pyrosequencing was performed on influenza A(H1N1) and A(H3N2) viruses to detect markers of adamantane resistance in the M2 gene. Detection of NAI-resistance was done by NA inhibition assay with chemiluminescent substrate (NAStar® kit). IC<sub>50</sub> values (the concentration of a drug needed to inhibit 50% of the virus NA activity) were determined (Robosage) and compiled with data from previous seasons. Viruses with very high IC<sub>50</sub> values were considered extreme outliers and subjected to NA sequence analysis in order to identify the genetic markers of NAI-resistance. The IC<sub>50</sub> values of the remaining viruses were statistically analyzed by (sub)type and drug to detect mild outliers (IC<sub>50</sub> > mean + 3 standard deviations).

### Enhanced Surveillance for Antiviral Resistance

During the 2007-08 influenza season, CDC requested submission of larger numbers of influenza A and B viruses, especially A(H1N1) viruses, from U.S. public health laboratories. The laboratories submitted virus isolates, original clinical specimens, or both (matched). At CDC, virus isolates were tested directly, without additional propagation, with the NA inhibition assay. To expedite detection of H274Y mutants, an NA pyrosequencing approach was developed and utilized to test grown viral isolates as well as original clinical specimens. The extracted viral RNA was also used to detect markers of M2 resistance by pyrosequencing.

**Results:** Overall, 1198 viruses isolated between October 1, 2007 and February 29, 2008 from 36 U.S. states and 22 foreign countries were screened for drug resistance. Seventy-one oseltamivir-resistant influenza A(H1N1) viruses with the H274Y were detected through NA inhibition assay ( $IC_{50}$  values > 100 nM) and/or NA pyrosequencing. To date, the frequency of resistance of oseltamivir resistance among A(H1N1) is 10.1% (71 of 702) in the U.S.; the prevalence of resistance varies among individual states from 0% to 24%. These data demonstrate a substantial rise in the frequency of H274Y mutants among 2007-08 influenza A(H1N1) viruses compared to previous seasons [0/53 (0%) in 2004-05; 1/258 (0.4%) in 2005-06; and 5/817 (0.6%) in 2006-07]. All oseltamivir-resistant A(H1N1) viruses with H274Y mutation tested so far are susceptible to zanamivir and to adamantanes. Among 199 influenza A(H3N2) and 181 influenza B viruses tested, no resistance to NAIs has been detected. Resistance to adamantanes remained high among influenza A(H3N2) viruses: 99.4% (154/155) of viruses isolated in the U.S. and 100% (36/36) of foreign viruses tested while the overall resistance to adamantanes for influenza A(H1N1) viruses was 12.1% (80/660) in the U.S. and 32.5% (38/117) in foreign viruses tested so far. Analysis of drug resistance for the 2007-08 season is ongoing.

**Conclusion:** The solicitation of additional viruses specifically for antiviral testing in conjunction with the conventional surveillance procedures was critical for providing a timely investigation of the unprecedented increase in oseltamivir resistance in the U.S. in the early 2007-08 season. The development of the NA pyrosequencing assay for established markers of resistance in the NA gene allowed for high-throughput and rapid testing of original clinical specimens, as well as timely reporting of results. Nevertheless, the detection of resistance to NAIs cannot be replaced entirely by NA pyrosequencing or other genetic methods because the current knowledge of markers of NAI resistance is insufficient.

6-013

### Arbidol-effective antiviral drug against influenza viruses

**Leneva, Irina<sup>1</sup>; Fedyakina, I.T.<sup>1</sup>; Burtseva, E.I.<sup>2</sup>**

<sup>1</sup>Centre of Chemistry of Drugs, Russian Federation; <sup>2</sup>Ivanovsky Institute of Virology, Russian Federation

An antiviral drug arbidol (1-methyl-2-phenyl-thiomethyl-3-carboxy-4-dimethylaminomethyl-5-hydroxy-6-bromo-indolehydrochloride monohydrate) has been shown to be effective in prophylaxis and treatment of influenza A and B with no side effects reported. Arbidol was shown to inhibit membrane fusion in vitro both between virus and plasma membrane and between virus and membrane of endocytic vesicles. The studies of arbidol effect upon replication of a panel of reassortants between A/Singapore/1/57(H2N2) and A/Chicken/Germany/27 (Weybridge strain, H7N7) showed that the greater sensitivity of the Weybridge virus to arbidol was determined by the HA gene, while there was no correlation between sensitivity to arbidol and any other viral genes. The arbidol-resistant viruses were generated through serial passages in the presence of increasing concentrations of arbidol in cell cultures. All mutants had amino acid substitutions at different positions in the HA2 subunit. We studied influenza A and B viruses collected from 25 patients before and during treatment with arbidol for their sensitivity to arbidol in MDCK cells and sequenced the HA genes. In our study it was found that no arbidol resistance had emerged during 5 days of therapy of acute influenza infection. The study of arbidol effect on viral replication showed that in MDCK cells arbidol inhibited reproduction of all antigen subtypes of human influenza A and B viruses (50% effective concentration  $EC_{50}$ , 3  $\mu$ g mL<sup>-1</sup> to 12  $\mu$ g mL<sup>-1</sup>). Arbidol efficiently inhibited in cell culture replication of avian influenza virus A/Hong Kong/156/97 (H5N1) which has caused human infection, non pathogenic A/H5 influenza viruses isolated in Russia from wild birds and highly pathogenic avian influenza H5N1 virus isolated from chickens in Novosibirsk, Russia ( $EC_{50}$ , 4  $\mu$ g mL<sup>-1</sup> to 30  $\mu$ g mL<sup>-1</sup>). Mice that were given arbidol at 10 and 50 mg per kg of body weight per day, were completely protected against challenge with pathogenic influenza avian H5N1 virus isolated from chickens.

6-014

# **Low penetration of oseltamivir and its carboxylate into cerebrospinal fluid in healthy Japanese and Caucasian volunteers**

**Boak, L.<sup>1</sup>;** Jhee, S.S.<sup>2</sup>; Yen, M.<sup>2</sup>; Ereshefsky, L.<sup>2</sup>; Leibowitz, M.<sup>2</sup>; Schulte, M.<sup>3</sup>; Kaeser, B.<sup>3</sup>; Patel, A.<sup>1</sup>; Prinssen, E.P.<sup>3</sup>; Rayner, C.R.<sup>3</sup>

<sup>1</sup>Roche Products Ltd, UK; <sup>2</sup>California Clinical Trials, USA; <sup>3</sup>F. Hoffmann-La Roche Ltd, Switzerland

**Background:** In recent years, and most commonly in Asian countries, abnormal or delirium-like behaviours have been reported with a low incidence in young individuals with influenza who were also receiving the antiviral prodrug oseltamivir. Although no causality could be demonstrated, these reports have generated renewed interest in the central nervous system (CNS) tolerability profile of oseltamivir. In the current study, we explored the CNS penetration of oseltamivir and its active metabolite oseltamivir carboxylate (OC) in healthy adult volunteers.

**Methods:** All participants in this single-centre, open-label study were healthy Caucasian or Japanese adults (aged 20–45 years). CNS penetration was assessed by determining the pharmacokinetics of oseltamivir and OC in plasma and cerebrospinal fluid (CSF) after a single oral dose of 150mg oseltamivir phosphate, mimicking the steady state plasma concentrations of the recommended 75mg twice daily oseltamivir regimen for adults. Oseltamivir and OC were measured in CSF and blood samples taken immediately before dosing and at 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 12, 20 and 24 hours after dosing (14 sampling points in total).

**Results:** Eight healthy male subjects (four Caucasian and four Japanese) were enrolled in the study (mean age [range]: 27.9 [24–35] years). Oseltamivir concentrations were notably lower in CSF than in plasma (mean of observed maximum concentrations [C<sub>max</sub>]: 2.4ng/mL vs 115ng/mL, respectively). OC concentrations were also lower in CSF than in plasma (mean of observed C<sub>max</sub>: 19.0ng/mL vs 544ng/mL, respectively). The corresponding mean C<sub>max</sub> CSF/plasma ratios were 2.1% for oseltamivir and 3.5% for OC. Overall exposure to oseltamivir and OC in CSF was also low versus plasma (mean area under the concentration-time curve [AUC] CSF/plasma ratio: 2.4% for oseltamivir and 2.9% for OC). No gross differences in the pharmacokinetics of oseltamivir or OC were observed between the Japanese and Caucasian subjects. Oseltamivir was well tolerated.

**Conclusions:** In the healthy adult Caucasian and Japanese volunteers investigated in this study, the CNS penetration of oseltamivir and OC was low. These findings are consistent with emerging data that suggest a low potential for oseltamivir or OC to induce or exacerbate CNS events in individuals with influenza; a disease-related effect appears likely.

6-015

# **A prospective study assessing the emergence and transmissibility of oseltamivir resistance following treatment of children with influenza**

**Stephenson, I.<sup>3</sup>;** Democratis, J.<sup>1</sup>; Lackenby, A.<sup>2</sup>; McNally, T.M.<sup>3</sup>; Ellis, J.S.<sup>4</sup>; Bermingham, A.<sup>4</sup>; Krommrower, D.<sup>3</sup>; Zambon, M.C.<sup>4</sup>

<sup>1</sup>Centre for Infections, Health Protection Agency/University Hospitals Leicester, UK; <sup>2</sup>Centre for Infections, Health Protection Agency, UK; <sup>3</sup>University Hospitals Leicester, UK; <sup>4</sup>Centre for Infections, Health Protection Agency, UK

**Background:** Significant resistance to oseltamivir (18%) has been reported in Japanese children with influenza treated using a weight adjusted daily dose of 4mg/kg. We treated children with a different dosing regime of oseltamivir and assessed emergence and transmission of antiviral resistance.

**Methods:** During the 2005–2007 influenza seasons, 77 children (1–12 years) with a positive influenza rapid test presenting within 48 hours of symptoms were prescribed oseltamivir. Nasopharyngeal swabs were taken before and at least once during or after treatment. Samples from household contacts were collected on day 9 (2006/07 season) and those positive by RT-PCR evaluated for transmission of resistance. Susceptibility of virus isolates to neuraminidase inhibitors (NAI) was assessed using IC50 determination and pyrosequencing.

**Results:** Of the 77 participants, 64 confirmed influenza positive by RT-PCR had pre- and post-treatment samples available for analysis. Viral subtyping indicated influenza B (19, 30%), A/H1N1 (11, 17%), A/H3N2 (34, 53%). Virus isolates were recovered from 58 (91%) pre-treatment samples, 42% samples collected between days 4–7 and in 1 sample collected between days 9–11. Phenotypic resistance was observed in 4 (6%) patients overall post-treatment. Resistance was found in 3/11 (27%) subjects with influenza A/H1N1 and 1/34 (3%) patients with influenza A/H3N2. No phenotypic resistance was noted in patients with influenza B. Pyrosequencing confirmed the resistant genotypes H274Y in H1N1, R292K in H3N2 and identified further resistant quasi-species in a small number of post-treatment samples without evidence of phenotypic resistance. No transmission of resistance to household contacts was found. No significant rises in viral load, determined by quantitative PCR, were seen in patients who developed resistance.

**Discussion:** Children shed detectable virus up to 11 days following treatment. Higher than expected levels of phenotypic resistance were found in treated children with H1N1 influenza despite increasing oseltamivir dosing. These findings are important in drug development and antiviral stockpiling strategies.



### A combinatorial antiviral approach against influenza A virus using ribozyme and siRNA

**Khanna, Madhu; M.K.**

*Department of Respiratory Virology, VP Chest Institute, India*

The seriousness of influenza as a disease and the safety of influenza drugs and vaccines are very important in limiting the impact of future pandemics of influenza. Recent advances in antisense technology have emerged as a ray of hope against many pathogens. The latest fad is siRNAs, but still these have their own limitations such as silencing off targets and no access to nuclear genes. Therefore, among the alternatives, catalytic nucleic acids are the best candidates. The aim of this study is to use catalytic nucleic acids such as Ribozymes and DNAzymes as candidates for having control over the replication of influenza virus in the host cells. The M1 genes of A/PR/8/34 (H1N1) strains were cloned in pcDNA 3. The computer-based secondary structures of RNA were analyzed to design the DNAzymes with 10-23 catalytic motifs and the hammerhead Ribozymes. These DNAzymes and Ribozymes were used for in-vitro cleavage of RNA of the corresponding M1 gene in the presence of different concentrations of MgCl<sub>2</sub>. The cleaved product were separated by PAGE and analyzed by autoradiography. The DNAzymes and Ribozymes were also used in combination. The DNAzymes were able to cleave the M1 RNA at 137 nt position whereas Ribozymes targeted at 163 nt position in the same target. These catalytic nucleic acids were highly efficient under the simulated physiological conditions. When DNAzymes and Ribozymes were used in combination, the cleavage was enhanced as compared to when they were used alone. siRNA-Ribozyme construct was designed, and when the siRNA-ARz construct was incubated with the cell extract, there was an efficient cleavage of the spacer sequences and both the siRNA and the Ribozyme could be seen as separate entities in in-vitro conditions. The modulation of the expression of the target gene in a controlled manner was shown at RNA level by RT-PCR and FACs. This combinatorial strategy could be used to design multi-target DNA-enzymes and Ribozymes to delay the appearance of escape mutants.

### Susceptibility of influenza isolates circulating in the Russian Federation to anti-influenza drugs

**Zarubaev, Vladimir; Meleshkina, I.A.; Shtro, A.A.; Guseva, V.M.; Erokin, M.Y.; Kononova, N.I.; Yaglovskaya, I.B.; Gudkova, T.M.; Pisareva, M.M.; Grudin, M.P.; Kiselev, O.I.**

*Influenza Research Institute, Russian Federation*

**Introduction:** Influenza represents a serious challenge for health protection services all over the world. In addition to potential high pathology to humans which is registered mostly during epidemics and pandemics as a sharp rise in morbidity and mortality, it is able to aggravate significantly the course of other respiratory and cardiovascular diseases and cause the suppression of immune systems often leading to chronic complications. The virus can also lead to transplacental penetration causing abnormalities in fetus development when exposed to the virus during the first trimester of pregnancy. This pathogen should therefore be considered one of the primary targets for development of antivirals and vaccines. Like other RNA-genome viruses, influenza virus has very high rate of mutations due to the lack of error-correcting activity in its polymerase complex. This results in gradual antigenic drift of surface glycoproteins leading, in turn, to a decrease in vaccine efficacy. Another consequence of the accelerated mutational process is fast selection of drug-resistant strains. According to the data of national surveillance systems, the portion of amantadine- and rimantadine-resistant strains reached 90% and higher in 2004-2005 with a slow decrease in 2006-2007. Recently, several influenza isolates appeared resistant to another anti-influenza drug oseltamivir carboxylate (Tamiflu). Wide spreading of such resistant strains may lead to inefficiency of therapy and prevention of influenza by synthetic antivirals. The surveillance for drug-susceptibility of circulating viruses is therefore an important part of the world influenza monitoring system. Here, we present the results of monitoring the drug-resistance of influenza viruses circulating in the Russian Federation in respect of two antivirals now used in clinical practice – rimantadine and Tamiflu.

**Materials and methods:** Viruses isolated from clinical specimens and previously subtyped with type-specific sera were serially diluted in a culture medium and grown in MDCK cells for 24 hours in the presence of rimantadine (1-10 microgram/ml) or oseltamivir carboxylate (0.2 – 25 microgram/ml). After the incubation, cells were fixed and the expression of virus-specific proteins was analyzed by an ELISA test with viral nucleoprotein-specific antibodies. The ELISA reaction was considered positive if optical density (OD<sub>450</sub>) exceeded the value of corresponding uninfected control cells by more than two times. Supernatant was analyzed for presence of the virus by a hemagglutination test by mixing with an equal volume of 1% guinea pig erythrocytes in round-bottomed wells. Virus titer was considered as reciprocal to the final dilution of the inoculum able to cause hemagglutination

in 50% of wells and expressed in 50% infecting doses (ED<sub>50</sub>), or, in the case of an ELISA test, as the reciprocal of the final dilution of the virus that generated a positive ELISA response. Strains were considered rimantadine-sensitive if optical density in wells infected with 10 50% infectious doses (ID<sub>50</sub>) in the presence of 1 microgram/ml of rimantadine was lower than that in control wells (without drugs) by two times or more, otherwise the strain was considered resistant. Resistance to Tamiflu was determined as the ability of a given isolate to decrease its infectious titer in the presence of 1 microgram/ml of oseltamivir carboxylate to 1 log<sub>10</sub> EID<sub>50</sub> or less compared to a control without drugs, otherwise the strain was considered Tamiflu-sensitive. Specific mutations responsible for drug resistance were localized in corresponding genes by PCR-sequencing of viral RNA.

**Results:** Among 73 strains isolated in 2008, 71 were of H1N1 subtype and 2 of H3N2 subtype. In the H1N1 group, 17 isolates appeared rimantadine-resistant (24%) and 54 - rimantadine-susceptible (76%). Of two H3N2 isolates, one (50%) appeared rimantadine-resistant and one (50%) - rimantadine-sensitive. In general, 25% of isolates were resistant to rimantadine. For 12 isolates, data of the virus-inhibition assay and ELISA were confirmed by sequence analysis by detection of specific mutation (S31N). This suggests a sharp decrease in the degree of rimantadine-resistance in the viral population in 2008 compared to 2007. Regarding susceptibility to oseltamivir, among 65 tested isolates, 41 (63%) appeared resistant to the compound while only 24 (37%) were drug-sensitive. The results obtained were confirmed for 12 strains by direct detection of the mutation in position 275 of the NA gene. Our data about significant increases in the number of influenza isolates resistant to neuraminidase inhibitors are in agreement with results of other studies having demonstrated the same tendency in virus evolution.

6-020

## Confronting an influenza pandemic with inexpensive generic agents

*Fedson, David S.*

*none, France*

Avian influenza A/H5N1 presents a serious and possibly imminent pandemic threat. If the pandemic virus should emerge within the next year or two, the world will have to depend on conventional adjuvanted inactivated H5N1 pandemic vaccines. Today, all vaccine companies could produce in a 6-month period (i.e., approximately 9 months after the emergence of the pandemic virus) enough doses of an adjuvanted, antigen sparing H5N1 vaccine to vaccinate with two doses for about 750 million people [1]. This number is less than the combined populations of the nine

countries that produce almost all of the world's seasonal influenza vaccines. Moreover, during this 9-month period, the pandemic virus will have already spread throughout the world. Currently, there is no logistical plan for distributing supplies of pandemic vaccines to the "have not" countries that will not be able to produce them. Many health officials have placed their hopes on stockpiles of antiviral agents. Recently, however, resistance to oseltamivir (Tamiflu) has emerged in seasonal H1N1 viruses, and this development has prompted concern that similar resistance could develop in a future pandemic virus. More importantly, government stockpiles of Tamiflu in "have not" countries would be sufficient to treat about 1% of the people who live in these countries. At a recent scientific meeting in Singapore, investigators reported that among people in Indonesia who had been infected with the clade 2 H5N1 virus, 33/33 (100%) of those who received no antiviral treatment died. It is not difficult to imagine that if this virus were to evolve into a human pandemic virus, we could see a global population die off. That such a virus could emerge through genetic reassortment was shown experimentally 35 years ago. The conclusion is inescapable: for the foreseeable future, our current "top down" approach to confronting a newly emergent pandemic virus will be incapable of providing timely supplies of affordable vaccines and antiviral agents to the more than 85% of the world's people who live in "have not" countries. The consequences could be disastrous. Given the overwhelming need for effective alternatives for pandemic treatment and prophylaxis, a "bottom up" approach using generic agents that target the host immune response should be considered [2,3]. Many influenza scientists doubt these agents would be effective. Nonetheless, retrospective studies suggest that statins improve outcomes in patients with bacteremia and reduce 30-day pneumonia mortality by approximately 50%. A preliminary report of results from a randomized controlled trial of statin treatment in 62 ICU pneumonia patients showed that hospital mortality was reduced by 51% ( $p = 0.026$ ). Statins might be similarly effective against influenza. Many pulmonary investigators believe that PPAR $\alpha$  and PPAR $\gamma$  agonists (fibrates and glitazones, respectively) could be used to treat acute lung injury. An important experimental study has shown that the fibrate gemfibrozil (a PPAR $\alpha$  agonist) reduced mortality in H2N2 influenza virus-infected mice by 54%. There is considerable molecular cross-talk between statins and PPAR agonists and combination therapy is safe. In patients with cardiovascular diseases, the clinical effects of statins and fibrates are additive. Generic agents with antiviral activity should also be considered [3]. Chloroquine increases endosomal pH and acts as an antiviral by impairing influenza virus release into the cytosol. Resveratrol, a polyphenol found in red wine, reduces influenza mortality in experimentally infected mice, probably by inhibiting the export of viral nucleoprotein from the nucleus. The many effects of catechins (found in green tea) and curcumin (turmeric in curry) on cell signal transduction suggest that they too might have anti-influenza activity. These generic agents have received virtually no attention from influenza scientists. All of these agents - statins, fibrates, chloroquine and resveratrol - and several others

are produced as generic medications in developing countries. They are very inexpensive, could be stockpiled and would be available on the first pandemic day. There is no guarantee that generic agents will be beneficial in a pandemic. Nonetheless, we have a choice. We can undertake the necessary laboratory and clinical research before the pandemic arrives, and perhaps discover that generic agents will not be useful. Alternatively, we can undertake the research after the pandemic has passed, only to discover that millions could have been saved. We can avoid this choice no longer. Most of the world's people lack realistic alternatives for confronting the next pandemic. For this reason, we cannot afford not to undertake the research needed to determine whether these and other generic agents could mitigate the effects of the next pandemic. Otherwise, we might face an unprecedented global public health crisis.

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6-021

### Emergence of oseltamivir resistant influenza A(H1N1) viruses in the Netherlands during the winter 2007/2008

**Meijer, A.<sup>1</sup>; Dijkstra, F.<sup>1</sup>; Donker, G.A.<sup>2</sup>; van Beek, R.<sup>3</sup>; Jonges, M.<sup>1</sup>; van der Sande, M.A.B.<sup>1</sup>; Boucher, C.A.<sup>3</sup>; Koopmans, M.P.G.<sup>1</sup>; Osterhaus, A.D.M.<sup>3</sup>; Rimmelzwaan, G.F.<sup>3</sup>**

<sup>1</sup>National Institute for Public Health and the Environment (RIVM), Bilthoven, Netherlands; <sup>2</sup>NIVEL, Netherlands Institute for Health Services Research, Utrecht, Netherlands; <sup>3</sup>Department of Virology, Erasmus Medical Centre (ErasmusMC), Rotterdam, Netherlands

**Background:** Continuous monitoring of influenza antiviral susceptibility has become more important since the introduction of the neuraminidase inhibitors (NAI) in 1999, in addition to the existing adamantane M2 channel inhibitors (M2I). Since the 2005/2006 winter season the Dutch National Influenza Centre (ErasmusMC and RIVM) systematically monitors the antiviral susceptibility of influenza viruses derived from patients with influenza-like illness or acute respiratory infection who consult a general practitioner of the influenza sentinel network (coordinated by NIVEL). Only sporadically were viruses with a lower susceptibility for the NAI found in the Netherlands in the winter seasons 2005/2006 and 2006/2007, whilst an increasing

proportion of A(H3N2) viruses were found resistant to M2I.

**Methods:** During the 2007/2008 season, influenza viruses detected in clinical specimens from sentinel and non-sentinel (through hospital and peripheral laboratories) patients were tested for antiviral susceptibility. Fifty percent inhibitory concentrations (IC<sub>50</sub>s) for the NAIs oseltamivir and zanamivir were determined using a fluorescent inhibition assay. Sequencing of the neuraminidase (NA) gene and the M2 ion channel gene was used to identify mutations previously associated with antiviral resistance to NAI and M2I respectively. To determine the possible impact of oseltamivir resistant A(H1N1) viruses on the severity of disease, basic descriptive variables and information on symptoms, complications and exposure to antivirals were collected.

**Results:** A(H1N1) influenza viruses (91% of all subtyped influenza A viruses) dominated the first part of the 2007/2008 winter season followed by type B viruses from week 9/2008 onward (Figure 1).

By the end of March 2008, 132 isolates [119 A(H1N1), 1 A(H3N2), 12 B] were analyzed for antiviral resistance. Resistant strains were only detected among the A(H1N1) viruses from sentinel and non-sentinel sources; 33 (28%) were found resistant and contained the H274Y substitution in the neuraminidase that has been associated with oseltamivir resistance previously. None of the oseltamivir resistant viruses tested for zanamivir and amantadine susceptibility were resistant to these antivirals. Further analysis of A(H1N1) viruses for which the NA nucleotide sequence was available showed that the resistant viruses (n=28) had glycine (G) at position 354, whereas the sensitive viruses (n=75) had aspartic acid (D) at that position. This substitution does not have known association with oseltamivir resistance.

By four-week period, the proportion of resistant viruses increased from 0% in weeks 40-51/2007 to 14% in weeks 52/2007-3/2008, 34% in weeks 4-7/2008 and 35% in weeks 8-11/2008. For a breakdown by week see Figure 1.

Basic descriptive variables were available for all 119 patients with the A(H1N1) virus and clinical data for 30 (all sentinel patients). The proportion of resistant strains among viruses from sentinel and non-sentinel patients was not significantly different, 20% and 30% respectively ( $X^2=1.196$ ,  $p=0.274$ ). Furthermore, no differences ( $p>0.05$ ) were found between patients infected with an oseltamivir resistant or sensitive virus with respect to age, gender, symptoms, complications or death. None of the 30 patients for which clinical data were available used oseltamivir prior to virus isolation.

**Conclusions:** Similar to many countries in Europe and worldwide, oseltamivir resistant A(H1N1) influenza viruses started to spread in the Netherlands during the 2007/2008 winter season with an overall prevalence of 28% among 119 A(H1N1) viruses analyzed by March 2008. Based on the current data, no differences in clinical impact between resistant and sensitive viruses were found. The analysis will be repeated when more data become available.

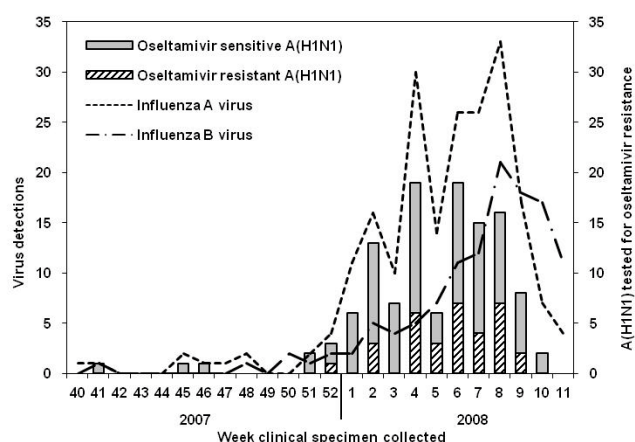


Figure 1. Time trends of type A and B influenza virus detections and of oseltamivir resistance of A(H1N1) influenza viruses during the 2007/2008 winter season in the Netherlands.

#### Acknowledgements:

We would like to thank all patients and their general practitioners or hospital physicians for providing information and specimens for investigation, the Dutch virologists for sending non-sentinel virus isolates to the NIC, and the Dutch Society for Clinical Virology for their help in designing the clinical study among non-sentinel patients.

6-022

### Increased prevalence of antiviral resistant influenza A viruses in Germany during the last seasons

Duwe, S.; Gravenstein, I.; Friedrich, M.; Schweiger, B.

National Reference Centre for Influenza, Robert Koch-Institut, Germany

The high frequency of adamantane resistant influenza viruses isolated worldwide and the emergence of neuraminidase inhibitor (NAI) resistant A/H1N1 viruses reveal the importance for monitoring the occurrence and spread of resistant viruses during seasonal epidemics. Therefore, rapid methods for genotypic analysis were developed and the influenza viruses circulating in Germany during the last ten seasons were examined for resistance-associated substitutions within their therapeutical target proteins M2 channel and neuraminidase.

RT-PCR assays with subsequent pyrosequencing (PSQ) for the detection of neuraminidase substitutions within the neuraminidase of A/H3N2 (119, 292, 294), A/H1N1 (274, 292, 294) and influenza B viruses (152, 198, 222, 250, 402) were designed. Positive controls created by site-directed mutagenesis were used for evaluation of these assays. Traditional sequencing methods were applied in addition to PSQ for determination of the genotype. A fluorometric neuraminidase activity assay was performed for phenotypic analysis. The RT-PSQ-assays discriminate clearly wild type virus from mutant virus and are appropriate to monitor the

incidence of mutant viruses.

Influenza A viruses circulating in Germany between October 1998 and May 2007 were susceptible to neuraminidase inhibitors. No resistance-associated mutation was detected within the neuraminidase genes of 475 representatively selected viruses. However, A/H1N1 viruses carrying the neuraminidase substitution H274Y that confer resistance to oseltamivir occurred in December 2007. At present, 46 out of 402 (11%) A/H1N1 viruses circulating in Germany during the 2007/2008 season showed resistance to oseltamivir. Adamantane resistant influenza viruses occurred in Germany for the first time in winter 2004/2005. After a dramatic increase from 12% resistant viruses (2004/2005) to 83% in the following season, a regressive trend in prevalence of resistant viruses (45%) has been detected for the last season (2006/2007). All resistant viruses belong to the subtype A/H3N2, while no M2 protein substitution was detected within A/H1N1 viruses. No influenza B viruses with reduced susceptibility to neuraminidase inhibitors were detected by analysing seasonal influenza viruses from October 2000 to the end of March 2008.

As the truly unexpected appearance of resistant A/H1N1 viruses has shown, an increase of resistant viruses seems to be likely. Moreover, a wider application of antivirals for treatment and prophylaxis of seasonal influenza infections may contribute to an increase in resistance. Thus it is important to perform antiviral susceptibility monitoring as part of the ongoing influenza surveillance.

6-023

### Oseltamivir resistant influenza A(H1N1) viruses persisted throughout the 2007/2008 season in Norway

Dudman, S.; Borgen, K.; Hauge, S.H.; Hungnes, O.

Norwegian Institute of Public Health, Norway

**Background:** The 2007/2008 influenza season in Norway was comparably mild and dominated by influenza A(H1N1) viruses belonging to the same main genetic lineage as the current vaccine strain A/Solomon Islands/3/2006. In January 2008, 12 out of 16 Norwegian A(H1N1) viruses tested for susceptibility to antiviral drugs in the VIRGIL reference laboratory in the UK turned out to be highly resistant to the neuraminidase inhibitor drug oseltamivir (1). This resistance trait is caused by a previously known histidine-to-tyrosine substitution at amino acid position 274 (H274Y) in the viral neuraminidase gene. However, this initial high proportion of resistance was unprecedented, and the possible ability of the current mutant viruses to compete well with the spread of similar, wild-type viruses would be a completely novel development with potentially considerable public health implications. Therefore, on January 25, we notified WHO under

the International Health Regulations of this high proportion of oseltamivir resistant influenza A(H1N1) virus. In order to investigate the epidemiological and clinical characteristics associated with this oseltamivir resistant influenza virus we initiated enhanced surveillance for the 2007/2008 season.

**Materials and Methods:** All influenza A viruses submitted to the National Influenza Centre from the 20 Norwegian microbiology laboratories and all influenza A viruses isolated from specimens taken at the approx. 70 influenza virus sentinel practices in Norway, were subtyped by PCR. All strains identified as A(H1) were tested for the relevant resistance mutation by sequence analysis. For all cases diagnosed with influenza A(H1) infection, we collected information on the clinical outcome and complications from the consulting physician using a structured questionnaire.

**Results:** From the end of March 2008, a conclusive result on resistance was obtained from 211 out of 259 A(H1N1) viruses. Of these 211 viruses, 143 (68%) were oseltamivir-resistant. The 211 patients ranged in age from 1 month to 70 years, and 111 (52%) were in the age group 25-59 years. 110 (52%) were male. Patients were from all parts of Norway. So far, information on clinical symptoms and complications has been obtained from approx 75% of the patients, with varying response rates for the different variables. Twenty-seven patients were admitted to hospital (age range 2 months to 47 years). There was no association between hospitalisation and being infected with a resistant virus: crude relative risk = 0.8 (95% CI 0.4-1.7). No fatal outcome was recorded. Eight of the patients had received antiviral drugs; all as treatment after the specimens were collected. One patient had been vaccinated against influenza. Eight of the patients had travelled abroad within one week prior to onset of the disease.

**Conclusions:** Oseltamivir-resistant influenza A(H1N1) virus persisted in Norway throughout the 2007/2008 winter outbreak, and maintained the same frequency as initially observed. This confirms the ability of the current H274Y mutant viruses to compete well with the spread of similar, wild-type viruses. The impaired fitness observed in H274Y mutant A(H1N1) viruses in previous seasons appears to have been overcome, probably by compensatory changes elsewhere in the virus genome. Preliminary data showed no difference in clinical outcome and complications between patients infected with oseltamivir resistant and susceptible A(H1N1) viruses. Final results will be presented at the conference.

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6-024

### Growth properties of influenza A H1N1 viruses from the UK resistant to neuraminidase inhibitors

**Thompson, C.I.; Lackenby, A.; Democratis, J.; Galiano, M.; Ellis, J.; Zambon, M.**

*HPA Centre for Infections, UK*

Influenza A H1N1 viruses resistant to the neuraminidase inhibitor Oseltamivir emerged globally during winter 2007-2008. Levels of circulation of the resistant variants ranged from 68% in Norway down to less than 1% in Italy, and were found in the UK at a level of 10%. The emergence of oseltamivir resistance did not correlate with drug use. Sporadic emergence of resistant H1N1 virus has been detected on only one occasion previously in the UK during the 2005-2006 winter influenza season. The 2007-2008 resistant strains were antigenically homologous to the dominant antigenic type A/Solomon Islands/3/2006 which circulated during this winter season. Oseltamivir resistance was identified by phenotypic testing and confirmed by identification of the 274Y mutation in N1 neuraminidase by sequence analysis. Prior to the 2007-2008 season, viruses harbouring this H274Y mutation were understood to have reduced replication efficiency and reduced transmissibility. We analysed the growth characteristics of a panel of temporally matched pairs of Oseltamivir sensitive and resistant viruses in two different cell lines. Paired viruses included examples from 2007-2008 season strains, in vivo emergence during drug treatment in an individual patient in the 2005-2006 season and sporadic emergence during the 2005-2006 season. Growth properties were assessed by infectious virus yield and quantitative PCR. Analysis of growth properties of a prototype resistant virus A/England/654/2007 revealed a growth advantage in a cell line genetically modified to over-express  $\alpha$ -2,6-linked sialic acid (SIAT cells) compared to standard MDCK cells. Resistant virus could be detected 8 hours earlier in SIAT cells compared to MDCK cells suggesting a receptor preference for  $\alpha$ -2,6-linked sialic acid found on SIAT cells. Comparison of growth curves in MDCK cells for A/England/654/2007 and a prototype Oseltamivir sensitive virus from the same season showed that resistant virus was slower to replicate in this cell line and time to reach peak titre was delayed by 24 hours compared to sensitive virus. In contrast, resistant virus isolated during drug treatment in the clinic had similar growth kinetics in both cell lines similar to the parental, drug sensitive strain. Resistant virus A/England/494/2006, a A/New Caledonia/20/1999-like virus isolated during the 2005-2006 season, showed similar growth properties in both MDCK and SIAT cells, whereas a geographically and temporally matched prototype sensitive virus grew significantly less well in SIAT cells, suggesting acquisition of  $\alpha$ -2,6-linked sialic acid binding ability by the resistant strain. Antigenic and sequence differences between the paired Oseltamivir sensitive and resistant viruses will also be discussed in the context of the growth properties.

6-025

# Monitoring influenza antiviral susceptibility in Portugal from 2004/2005 to 2007/2008 winter seasons

Correia, V.; Rebelo-de-Andrade, H.; Santos, L.; Gíria, M.

Centro Nacional da Gripe, Instituto Nacional de Saúde, Portugal

**Background:** Clinical use of specific antiviral agents in the prevention and treatment of influenza has started to receive greater attention in the last few years. This increase of interest for the antiviral therapy has been a result of the threat of an A(H5N1) influenza pandemic and of the possible non-existence of a specific vaccine during its initial months, which has lead several countries to stockpile antivirals as part of their pandemic preparedness plans.

Two classes of antiviral agents are currently available, (1) the adamantanes (amantadine and rimantadine) and (2) the neuraminidase inhibitors (oseltamivir and zanamivir). Since the 2002/2003 winter season, an increasing incidence of A(H3N2) influenza virus resistant to adamantanes has been detected worldwide. To a lesser extent, in the 2007/2008 winter season, the emergence of oseltamivir resistant A(H1N1) influenza virus started to be identified, particularly in Europe. The emergence of influenza resistant viruses in the community highlights the importance of global surveillance of antiviral resistance, for which the virological and epidemiological antiviral data collected worldwide is crucial.

The main objectives of this study are: (1) to establish an active and sustained national monitoring program on influenza antiviral susceptibility, using the tools and partnerships structured by the National Influenza Centre at national level; and (2) to evaluate the susceptibility to oseltamivir and amantadine of influenza viruses circulating in Portugal from the 2004/2005 to the 2007/2008 winter seasons. The accomplishment of these two objectives will allow for (1) improving the clinical and virological surveillance carried out in Portugal by the National Influenza Centre, and (2) contributing to global surveillance of antiviral resistance and to risk assessment of clinical use of the drug.

**Material and Methods:** Antiviral susceptibility testing was performed on influenza viruses isolated from respiratory specimens collected through the National Influenza Surveillance Programme. Since the 2005/2006 winter season, information regarding the exposure to and use of antivirals started to be included in the notification forms sent through the network of sentinel medical practitioners and the network of emergency units. Susceptibility to oseltamivir was initially evaluated by a phenotypic fluorescence assay in all the 270 viruses available, isolated from the 2004/2005 to the 2007/2008 winter seasons, by determining the IC<sub>50</sub> value (50% inhibitory concentration) for each virus. Subsequently, neuraminidase gene sequencing was performed in all statistical outliers and in 30% of the susceptible viruses, identified by fluorescence. Resistance to amantadine

was evaluated by a pyrosequencing search for specific mutations in 128 of the available viruses isolated from the 2004/2005 to the 2006/2007 winter seasons.

**Results:** Antiviral therapy is rarely used for the management of influenza in the community in Portugal (1.6% of the notifications received) and has been reported to be administrated only to adults and the elderly. The presence of influenza viruses was detected in only 8 (34.8%) of the 23 cases referring antiviral usage.

All 89 influenza A(H3N2) and 103 influenza B isolates analysed were shown to be susceptible to oseltamivir. For influenza B, the 3 outliers identified (1 in 2004/2005 and 2 in 2005/2006) did not exhibit different amino acid changes in the NA sequence than those observed in the other circulating B strains. Of the total 54 A(H1N1) isolates tested, 4 (16.7%) of the 24 from the 2007/2008 winter season revealed resistance to oseltamivir by exhibiting fluorescence IC<sub>50</sub> values approximately 300 to 500 times higher than the median value and by carrying the amino acid change H274Y in their NA sequence. None of these A(H1N1) seasonal resistant virus were directly associated with the use of antiviral drugs. However, 3 of the 4 resistant virus identified had the same geographic origin, a fact that is still under study.

Regarding amantadine, all 23 A(H1N1) isolates analysed by pyrosequencing were found to be susceptible. Resistance to this drug was detected in 24 (22.9%) of the 105 A(H3N2) isolates tested (specifically in the single isolate from 2005/2006 and in 23 (65.7%) of the 35 isolates from 2006/2007), by identification of the point mutation S31N in their M2 protein sequence. None of these seasonal amantadine resistant viruses are associated with cases in which antiviral drug use was notified.

**Discussion:** The low prescription of antiviral agents for the management of influenza identified in this study is in agreement with the situation observed in several other European countries. Although antivirals are being rarely used, the monitoring of antiviral resistance is essential since the emergence of resistant influenza viruses can be a result not only of drug use but also of spontaneous mutations or of the introduction or spread of resistant viral strains.

The origin of the A(H1N1) oseltamivir resistant virus in Portugal during the 2007/2008 winter season, and concurrently in several other European countries and in North America, Australia, China and Japan, is still unclear. A continuous national and worldwide monitoring of antiviral resistance in the following winter seasons will prove crucial to understanding (1) the persistence of these resistant strains throughout influenza seasons, (2) the clinical illness associated, and (3) its effect on viral evolution and on the effectiveness of drug use.

The emergence of amantadine influenza resistant virus in Portugal, first identified in the 2005/2006 winter season and persistent through the 2006/2007 winter season, is probably derived from the global spread of A(H3N2) virus bearing the mutation S31N. This global spread was possibly a consequence of an extensive drug use, predominantly in South-East Asia.

**Conclusion:** These national preliminary findings, although

restricted to the available sample size and technology capacity, presently contribute to the global surveillance and understanding of influenza antiviral drug resistance, on which the development of guidelines and recommendations for the use of antivirals in the management of influenza is based.

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6-026

### **Emergence of A (H1N1) influenza viruses resistant to oseltamivir during the 2007/2008 season: spatio-temporal trend and clinical impact in France**

Mosnier, A.<sup>1</sup>; Escuret, V.<sup>2</sup>; Enouf, V.<sup>3</sup>; Daviaud, I.<sup>1</sup>; Valette, M.<sup>2</sup>; Ferraris, O.<sup>2</sup>; Rameix-Welti, M.A.<sup>4</sup>; GROG, I.<sup>1</sup>; Cohen, J.M.<sup>1</sup>; Lina, B.<sup>2</sup>; **van der Werf, S.<sup>3</sup>**

<sup>1</sup>Réseau GROG, France; <sup>2</sup>NIC (South France), HCL & UCBL-CNRS FRE 3011, France;

<sup>3</sup>NIC (North France), Institut Pasteur, France; <sup>4</sup>Institut Pasteur, France

During the 2007/2008 winter period, A(H1N1) viruses resistant to oseltamivir have been observed in various regions of Europe and the Northern Hemisphere. This resistance was the consequence of the emergence of viruses displaying a H274Y mutation in the neuraminidase (NA) protein. During this epidemic, A(H1N1) was the most prevalent subtype. The emergence of the resistant virus was first detected in Europe (Norway), and rapidly, numerous countries reported resistant strains. The percentage of resistant isolates varied among countries, ranging in Europe from 0 to more than 66%. In France, the first resistant isolate was detected in November (week 45/07). The circulation of the virus was at epidemic levels between weeks 2 and 12 of 2008. During the surveillance period, more than 1300 viruses were isolated in France (data available in week 12/08). Among these, 526 influenza A(H1N1) viruses were tested for their susceptibility to Neuraminidase inhibitors showing resistance in 219 strains. The spatial distribution of these resistant isolates was not homogeneous in the country. There was a North/South and a West/East gradient. The largest proportion of resistant strains was identified in the North West part of France (57%), and the smallest in the South East part (31%). There was a trend in antiviral resistance over the period of circulation of the virus. During weeks 48/07 to 50/07, before the beginning of the epidemic, the rate of resistance was approximately 20% for the whole country. During weeks 51/07 to 6/08, the rate of resistance was approximately 40%, and at the end of the epidemic, the rate rose to up to 55%. This increase in the proportion of resistant viruses was observed in all regions, whereas the impact of influenza was equivalent in the various regions. The molecular follow-up of the

resistant strains showed the same mutations as described for viruses from other countries. Their NA gene segment displayed the H275Y mutation associated with oseltamivir resistance. This mutation was associated with the D354G mutation in most viruses. Phenotypically, these viruses had IC<sub>50</sub> values ranging from 250 nM to 1750 nM. No resistance to zanamivir has been observed. During the epidemic, we collected clinical information from patients presenting with virologically confirmed influenza infection. Overall, 366 cases have been analyzed including 161 patients with a resistant virus. This clinical evaluation was carried out at the onset of the disease and was concomitant with the collection of the swab specimen. The sex ratio of both groups of patients (sensitive vs resistant) was not statistically different (1.3 vs 1.1), nor was the age-range (0-81 vs 0-70). The mean and median age (19 vs 21 and 12 vs 17, respectively) were not statistically different between the two groups of patients. Clinical signs and symptoms of the two groups of patients were indistinguishable; there was no difference in the clinical presentation or in the severity. This emergence of A(H1N1) viruses naturally resistant to oseltamivir was monitored by the community- and hospital-based networks. There was no difference in the rate of detection of sensitive vs resistant viruses in the hospitalized patients as compared to the community setting. Overall, the resistant virus was responsible for numerous clinical cases and had the same apparent fitness and distribution as the sensitive virus. According to our data, the clinical impact of this emergence was minor. The reasons for the spatio-temporal gradient observed during the 2007/2008 epidemic remains to be elucidated.

## 7 GENETIC AND ANTIGENETIC EVOLUTION

7-001

### Rapid evolution of influenza A (H3N2) viruses and challenges in vaccine strains selections: U.S. influenza virologic surveillance, 2007-08 season

**Xu, X.;** Garten, R.J.; Balish, A.L.; Foust, A.S.; Veguilla, V.; Barnes, J.R.; Myrick, A.D.; Sessions, W.M.; Kocher, G.A.; Guo, L.; Mabry, J.E.; Wallis, T.C.; Hall, H.E.; Smith, C.B.; Shaw, M.W.; Cox, N.J.; Klimov, A.

*Influenza Division, NCIRD/CCID, Centers for Disease Control and Prevention, Atlanta, GA, USA*

**Introduction:** The H3N2 subtype of influenza A virus has been circulating in humans since its emergence in 1968. Viruses of this subtype are of particular importance because of the regular occurrence of excess pneumonia and influenza mortality during their circulation. Vaccination remains the most effective tool to reduce the impact and burden of influenza infections. Because of the rapid evolution of influenza viruses, the vaccine has to be evaluated annually to ensure that vaccine strains match the dominant circulating strains. Under current regulations, only those viruses which are isolated and propagated in embryonated eggs can be used for the production of inactivated trivalent influenza vaccine. In recent years, it has become increasingly difficult to isolate influenza A (H3N2) viruses in eggs. Rapid variation and difficulties in obtaining appropriate egg isolates have become major challenges for influenza A (H3N2) vaccine strain selections.

Influenza surveillance is essential for monitoring emergence and spread of antigenic variants. As the US National Influenza Center and one of four WHO Influenza Collaborating Center, the Influenza Division at CDC conducts comprehensive antigenic and genetic analyses of influenza viruses isolated from the US and other countries worldwide. Data obtained from virologic surveillance provide information vital for the WHO and FDA evaluation of current vaccine and for the selection of the most appropriate strains for the next influenza season.

**Material and Methods:** Influenza viruses received by CDC were propagated in MDCK cells or in the allantoic cavity of 9-11 day old embryonated eggs depending on the substrate used for primary isolation.

Viruses were antigenically characterized by hemagglutination inhibition (HI) tests using post-infection ferret antisera and turkey and/or guinea pig red blood cells.

Nucleotide sequences of hemagglutinin (HA) and neuraminidase (NA) genes from a subset of viruses were obtained using published protocols. Sequence analysis was performed as described previously.

Human serum samples collected from volunteers pre- and post-

vaccination were evaluated in HI and microneutralization tests.

**Results:** Although influenza A (H1N1) viruses predominated through mid-January 2008 in the US, influenza A (H3N2) viruses were reported more frequently than H1N1 viruses starting from week 4 of 2008. Beginning at week 6, influenza A (H3N2) viruses became the dominant influenza virus overall nationally.

Antigenic analyses revealed that only a small proportion of H3N2 viruses analyzed were well inhibited by ferret antiserum to A/Wisconsin/67/2005, vaccine strain for the 2007-08 influenza season. The majority of H3N2 viruses were well inhibited by ferret antiserum to a minor antigenic variant A/Brisbane/10/2007 virus, one of the few recent H3N2 egg isolates.

Sequence analyses indicated that the HA genes from recent H3N2 isolates fell into four major genetic lineages represented by A/Brisbane/10/2007, A/Nepal/921/2006, A/Henan/Jinshui/147/2007 and A/British Columbia/1287/2007, respectively. Since March 2007, the majority of US and worldwide viruses have belonged to the A/Brisbane/10/2007 lineage which is characterized by changes at amino acids 50 (glycine to glutamic acid) and 140 (lysine to isoleucine). Position 140 is located in the previously defined antigenic site A of H3 HA. A small number of recent US isolates belong to the A/Henan/Jinshui/147/2007 lineage with the characteristic amino acid change N144D, also located in the antigenic site A. A new lineage represented by A/British Columbia/RV1287/2007 recently emerged and shares 5 amino acid changes at positions 50 (glycine to glutamic acid), 140 (lysine to glutamine), 144 (asparagine to aspartic acid), 158 (lysine to arginine) and 310 (lysine to arginine). One virus isolated from the USA belonged to this lineage. Viruses of A/Nepal/921/2006 lineage have not been detected in the US since early 2007.

Data obtained from human serology tests revealed that post-vaccination antibodies to vaccine strain A/Wisconsin/67/2005 could not inhibit representative viruses A/Brisbane/10/2007 or A/Uruguay/716/2007 with a greater than 50% reduction of HI and/or microneutralization titers.

**Conclusions:** Influenza vaccine selection, especially for the H3N2 subtype, continues to be challenging. Data obtained from the US surveillance system during the 2007-08 influenza season detected antigenic drift among currently circulating influenza A (H3N2) viruses. The majority of circulating H3N2 viruses were more closely related to the A/Brisbane/10/2007 virus antigenically and genetically than to the vaccine strain A/Wisconsin/67/2005. Based on similar data collected internationally through the WHO Global Influenza Surveillance Network (GISN) and other WHO Influenza Collaborating Centers, WHO recommended the inclusion of an A/Brisbane/10/2007-like virus as the H3N2 vaccine component for the 2008-09 Northern Hemisphere influenza season.

### Molecular characterization and phylogenetic analysis of human influenza A viruses in 3 consecutive seasons with different epidemiological profiles

**Pariani, E.<sup>1</sup>; Frati, E.R.<sup>1</sup>; Amendola, A.<sup>1</sup>; Zappa, A.<sup>1</sup>; Bianchi, S.<sup>1</sup>; Colzani, D.<sup>1</sup>; Canuti, M.<sup>1</sup>; Anselmi, G.<sup>1</sup>; Zanetti, A.<sup>2</sup>; Tanzi, E.<sup>2</sup>**

<sup>1</sup>Dept. Public Health-Microbiology-Virology, Univ. Milan, Italy; <sup>2</sup>Dept. Public Health-Microbiology-Virology, Univ. Milan and CIRI-IV, Univ. Genoa, Italy

As part of the Italian Influenza Surveillance Network, the influenza activity and circulation of influenza viruses were observed in Lombardy (the most populous Italian region) during 3 consecutive seasons (2005/06-2007/08) outlined by different epidemiological profiles. The molecular characteristics of circulating A influenza viruses were analyzed to evaluate the introduction of new variants in the territory. Moreover, a phylogenetic analysis was carried out to comprehend the evolutionary features of A/H3N2 and A/H1N1 viruses in such different epidemiological scenarios.

**Methods:** The molecular characterization of 38 isolates, namely 20 A/H3N2 and 18 A/H1N1 influenza strains from 2005/06, 2006/07 and 2007/08 seasons was performed by sequence analysis of the globular head region of the HA protein (HA1 subunit), specific for influenza virus A/H3 (nt. 174-1056) and A/H1 (nt. 76-1090). Viral RNA was extracted from respiratory samples collected from outpatients with clinical evidence of influenza-like illness (ILI), during the 3 considered seasons, by the QIAmp Viral RNA kit (QIAGEN, GmbH, Germany). Following the RT-PCR of the HA1 gene, amplicons were purified and the nucleotide sequences were obtained from automated DNA sequencing on the genetic analyzer ABI PRISM 3100 (Applied Biosystem, CA, USA). Multiple sequence alignment was conducted using ClustalX, version 1.81. Phylogenetic trees from A/H1 and A/H3 HA1 sequences were constructed by means of the Neighbor-Joining method and Kimura 2-Parameter model, using the MEGA package, version 4.0. A bootstrap analysis (n=1000) was performed and major branches with bootstrap values >70% were identified as distinct groups. The HA gene sequences of the reference viral strains used in the construction of phylogenetic trees were obtained from the NCBI Influenza Virus Sequence Database. To estimate the selection pressures acting on the HA gene, codon-specific dN and dS values were inferred using the Nei-Gojobori method and Jukes-Cantor model, by MEGA, and the Single Likelihood Ancestor Counting (SLAC), Fixed Effects Likelihood (FEL) and Random Effects Likelihood (REL) methods, all incorporating the HKY85 substitution models with phylogenetic trees inferred using the Neighbor-Joining method, available at the Datamonkey facility.

**Results:** The HA sequences of the A/H3N2 viruses isolated in recent seasons fell into 4 principal phylogenetic groups as shown in Figure 1. Three viruses characterized during the 2005/06 season fell within the older phylogenetic group (Clade I) represented by A/Queensland/55/05 and characterized by the V112I and K173E

amino acid changes. Variants, represented by these viruses, were distinguished by an additional S199P substitution. The other A/H3 viruses isolated in the 2005/06 season were characterized by S193F and D225N changes in HA1 and were represented by A/Wisconsin/67/05 (Clade II). In addition, the majority of HA sequences of the A/H3N2 viruses isolated in the 2006/07 season fell within 2 principal clades, distinguished by the amino acid changes relative to A/Wisconsin/67/05. Several viruses fell within the Clade III, characterized by the G50E and K140I amino acid changes, and represented by A/Brisbane/10/07. Finally, the majority of the A/H3 viruses isolated during the 2006/07 season fell within Clade IV represented by A/Nepal/921/06 and characterized by the amino acid changes T128A, which resulted in the loss of a potential N-linked glycosylation site, R142G, L157S and K173E. Variants were further distinguished by amino acid substitutions V112I, P169S and R269K. There was no evidence of positive or negative selection in the sequence alignment of A/H3 viruses. The [dN-dS (+/- S.E.)] value was [-0.016 (+/- 0.006)]. The integrative selection analysis (SLAC p-value=0.; FEL p-value=0.1 and REL BF=50) found 1 positive selected codon in position 186 (epitope B) and 3 negative selected codons in position 222, 267 and 302, respectively. The HA sequences of the A/H1N1 viruses isolated during the 3 seasons were separated into 3 phylogenetic groups as shown in Figure 2. Clade I was characterized by the amino acid change Y252F, relative to the HA sequence of A/New Caledonia/20/99. All the A/H1N1 viruses isolated during the 2005/06 season fell in this clade. Three sequences presented the amino acid changes T82I and F260L whilst the rest were characterized by the R188K and N245S substitutions. The A/Milan/10/06 sequence was characterized by the amino acid change S161F which resulted in the loss of a potential N-linked glycosylation site. HA sequences within Clade II were represented by A/Solomon Islands/3/06, characterized by 5 amino acid changes T82K, Y94H, K140E, R208K and T266N, and exhibited a greater diversity. All the HA sequences, but one, of the viruses isolated during the 2006/07 season fell into this phylogenetic group and presented 3 additional amino acid changes, i.e. D35N, R188K and E273K. All the A/H1N1 viruses isolated during the 2007/08 season fell in Clade III and were represented by A/Brisbane/59/07. These sequences did not present any amino acid changes. The sequence alignment of A/H1 viruses reported a negative [dN-dS (+/- S.E.)] value [-0.060 (+/- 0.012)]. The integrative selection analysis found 4 negative selected codons in position 100, 113, 157 and 218, respectively.

**Conclusions:** A medium-low activity of influenza marked the last 3 influenza seasons in the studied area. During the 2005/06 winter season, influenza activity was patchy and the epidemic wave was sustained almost exclusively by influenza A viruses, accounting for 80.5% of total detections (51.7% A/H1N1 and 48.3% A/H3N2). The following winter season (2006/07) was dominated by influenza A/H3N2 viruses, accounting for 93.6% of total detections. The epidemic in the most recent influenza season (2007/08) was upheld by A/H1N1 viruses (40% of total detections) along with B viruses (60% of total detections). The

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first 2 seasons presented the co-circulation of A/H3 viruses belonging to distinct phylogenetic groups while in the latter season no A/H3 viruses were detected in the considered area. Overall, the A/H3 viruses analyzed presented heterogeneity in their HA sequences suggesting that several amino acid mutations were fixed in the viral population and a miscellaneous set of variants co-circulated notwithstanding the mid-low activity of influenza. While A/H1N1 viruses isolated during the 2005/06 season were mostly closely related to A/New Caledonia/20/99, the HA sequences of the A/H1N1 viruses from the 2006/07 exhibited a greater diversity and were A/Solomon Islands/3/06-like. The last season was characterized by the exclusive circulation of A/H1 viruses, all closely related to A/Brisbane/10/07-like ones. Overall, we observed that A/H3 viruses were characterized by the co-circulation of several variants up to 2 years and then almost completely substituted by new emerging variants with different antigenic features. A/H1 viruses present a more homogeneous circulation where a specific lineage clearly distinguishes each season. Despite the mid-low clinical activity of influenza during the 3 analyzed seasons, the molecular characterization of the viruses highlighted a considerable heterogeneity in their HA sequences suggesting the co-circulation of a miscellaneous set of variants.

Figure 1. H3 HA1 phylogenetic tree. There were a total of 876 positions in the final dataset. Triangles label the sequences from 2005/2006 and circles the ones from 2006/2007. Major amino acid changes are reported in block letters.

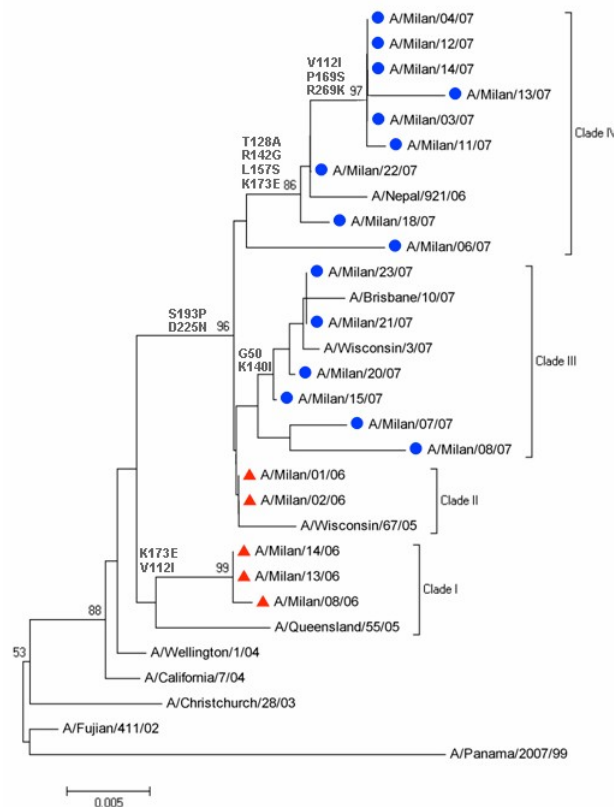
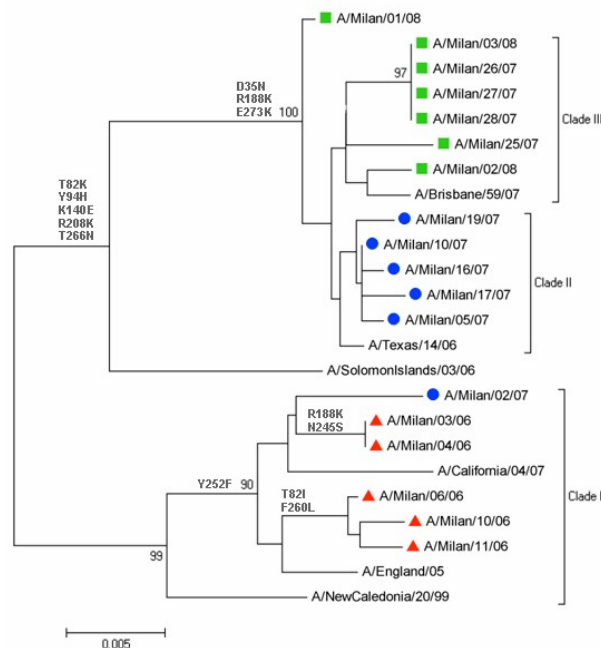


Figure 2. H1 HA1 phylogenetic tree. There were a total of 915 positions in the final dataset. Triangles label the sequences from 2005/2006, circles the ones from 2006/2007, and squares the ones from 2007/2008. Major amino acid changes are reported in block letters.





### Genetic evolution, antigenic drift and antiviral resistance among 2007-08 influenza A(H1N1) viruses isolated in the U.S.

**Garten, R.;** Foust, A.; Okomo-Adhiambo, M.; Sheu, T.; Deyde, V.; Barnes, J.; Myrick, A.; Smith, C.; Balish, A.; Kocher, G.; Sessions, W.; Gubareva, L.; Xu, X.; Klimov, A.

*Influenza Division, Centers for Disease Control and Prevention, USA*

**Introduction:** Influenza A(H1N1) viruses have been circulating in humans globally since their re-emergence in 1977. Vaccination is the most effective tool to reduce the impact and burden of influenza infections and the vaccine formulation has to be evaluated annually to ensure vaccine strains match the dominant circulating strains. For the first time in three years, H1N1 viruses predominated in the 2006-07 U.S. influenza season. Antigenic drift during 2006-07 replaced A/New Caledonia/20/1999, the vaccine strain in 2001 through 2006, with A/Solomon Islands/03/2006 recommended by the WHO as the H1N1 component in the 2007-08 Northern Hemisphere influenza vaccine.

The hemagglutinin (HA) antigen is the major component of the influenza vaccine and thus a focus of evolutionary analysis. Since 2001, two major genetic clades (1 and 2) of the HA genes were identified in circulating H1N1 strains worldwide. During the 2006-07 U.S. influenza season, the majority of isolates had an HA from clade 1. Viruses with the HA from clade 2 were more prevalent outside of the U.S. and several isolates from this clade shared a change at position Lys-144 to Glu, resulting in a decrease in hemagglutination inhibition (HI) by ferret antiserum raised against the A/New Caledonia/20/1999 vaccine strain. Within clade 2 HA, several subclades with distinct amino acid (aa) changes were identified.

Analysis of 2006-07 influenza A(H1N1) viruses for their resistance to licensed antivirals detected a small proportion (6%) of strains resistant to adamantanes (M2 blockers: amantadine and rimantadine). Nearly all were in one clade 2 subclade. During the 2007-08 season, the proportion of influenza A(H1N1) viruses resistant to M2 blockers was high in some Asian countries and had increased in the U.S. (to 12%) compared to the 2006-07 season.

A new antigenic variant of influenza A(H1N1) viruses within clade 2, A/Brisbane/59/2007, appeared in 2007-08, expanding the previously minor subclade 2b. Although resistance to neuraminidase-inhibitors (NAI) (oseltamivir and zanamivir) was rarely detected in either clade 1 or 2 viruses in previous years, a significant increase in the proportion of oseltamivir-resistant viruses was detected in the 2007-08 season in the U.S. as well as worldwide.

The aim of this study was to investigate the relationship between the antigenic evolution of recent influenza A(H1N1) viruses and their susceptibility to antivirals through full genome analysis of

strains isolated during the 2007-08 U.S. influenza season.

**Methods:** Influenza viruses received by the CDC were propagated in MDCK cells or in the allantoic cavity of 9- to 11-day-old embryonated chicken eggs depending on the substrate used for primary isolation. Viruses were antigenically characterized by HI tests using reference post-infection ferret antisera and turkey red blood cells. Serological assays with human serum samples were collected from volunteers pre- and post vaccination by HI tests. All viruses were tested in the chemiluminescent NA inhibition assay to detect zanamivir and oseltamivir resistance. In addition, viruses were analyzed by pyrosequencing of the M2 gene for the presence of established markers for resistance to adamantanes. Sequences of full and partial genomes of H1N1 viruses were obtained using protocols published previously. Phylogenetic analyses were performed using MEGA 4.1 and GARLI 0.96b7 software.

**Results:** Influenza A(H1N1) viruses continued to be the dominant subtype of influenza A viruses circulating in the U.S. from October 2007 until mid-January 2008, after which influenza A(H3N2) prevailed. The majority of H1N1 viruses were well inhibited by ferret antiserum to A/Solomon Island/03/2006, the 2007-08 vaccine strain. However, over the season an increasing number of isolates demonstrated decreased reactivity with the A/Solomon Island/03/2006 antiserum. These low reacting isolates were well inhibited by ferret antiserum raised to A/Brisbane/59/2007. Unlike previous seasons, clade 1 H1N1 viruses were rarely detected, instead clade 2 viruses predominated. The HA gene of 2007-08 clade 2 viruses evolved into three subclades (2a-c). The same subclades were also represented in the evolutionary tree of the NA gene. Subclade 2a viruses included the 2007-08 vaccine strain A/Solomon Island/03/2006, but did not circulate widely in the current season. Subclade 2b, represented by reference strains A/Brisbane/59/2007 and A/South Dakota/06/2007, became dominant in the U.S. Subclade 2b viruses shared AA changes in the HA at positions Asp-45 to Asn, Lys-149 to Arg, Arg-192 to Lys and Lys-276 to Glu. Three of the four characteristic AA changes were located in previously defined HA antigenic sites E, B and C, respectively. A small number of viruses bearing subclade 2c HA were seen in the U.S., while the majority were from South-East Asia. These isolates shared AA changes at positions Ser-46 to Asn, Arg-192 to Met, Ala-193 to Thr, and Thr-197 to Lys. Positions 192 and 197 are located in HA antigenic site B. Viruses with reduced HI titer against ferret antiserum to A/Solomon Islands/03/2006 were found in both 2b and 2c groups. In addition, data obtained from human serology tests revealed that post vaccination antibodies raised to the vaccine strain A/Solomon Islands/03/2006 could not inhibit the representative viruses A/Brisbane/59/2007 or A/South Dakota/06/2007 well, with approximately 50% reduction of HI titer, confirming the appearance of a new antigenic variant of influenza A(H1N1) viruses.

Full genome sequence analysis demonstrated that subclade 2b and 2c viruses were different genotypes but shared similar PB2 and PA genes. Unique patterns of antiviral resistance were identified in both genotypes. The majority of viral genomes with

a 2c HA possessed an M2 gene with a marker of resistance to adamantanes (Ser-31 to Asn). Beginning in late October 2007, an increase in the prevalence of oseltamivir resistance was detected in isolates collected from individuals without prior history of antiviral use. Oseltamivir-resistant viruses belonged only to the 2b subclade and contained an AA change of His-274 to Tyr in the NA. 2b viruses also shared AA changes in the NA at positions His-49 to Asn, Lys-82 to Glu, Arg-248 to Lys, Thr-286 to Ile, Lys-329 to Glu and Gly-357 to Asp. Position 248 is located in the vicinity of the NA active site. More recent oseltamivir-resistant isolates shared a reversion at AA Asp-357 to Gly.

**Conclusions:** The increase in global circulation of influenza A(H1N1) viruses since 2006 has created challenges for vaccine selection and raised concerns over optimal treatment strategies available for the control of influenza infections. Evidence of antigenic drift in recent circulating H1N1 strains has been detected in the present study and by other participants of the WHO Global Influenza Surveillance Network. This has resulted in the WHO recommending a change in the H1N1 vaccine component for 2008-09 Northern Hemisphere influenza season replacing A/Solomon Islands/03/2006 with an A/Brisbane/59/2007-like virus.

Full genome analysis found two major co-circulating genotypes with hemagglutinins from either subclade 2b or 2c. Subclade 2c viruses were predominantly adamantane-resistant while subclade 2b remained adamantane-sensitive. The appearance of oseltamivir resistance among recent 2b viruses was preceded by an AA change at position Arg-248 to Lys in the vicinity of the NA active site. This change may be a prerequisite for the fitness of the oseltamivir-resistant mutants bearing the His-274 to Tyr mutation.

Full genome analysis provides additional insights into a possible role of non-surface genes in the evolutionary advantage of particular antigenetic variants. It is also instrumental in the detection of reassortment events leading to an emergence of new genotypes which may affect viral fitness and confer an evolutionary advantage over other circulating viruses.



7-004

# **Biochemical and evolutionary characteristics of clinical influenza viruses H3N2 isolated in the Moscow region**

**Vorobjeva, Irina<sup>1</sup>; Poyarkov, S.V.<sup>1</sup>; Saphonova, O.A.<sup>2</sup>; Malyshev, N.A.<sup>2</sup>; Ovcharenko, A.V.<sup>1</sup>; Zhirnov, O.P.<sup>1</sup>**

<sup>1</sup>D.I. Ivanovsky Institute of Virology, Russian Federation; <sup>2</sup>Moscow Infection Clinics No.1, Russian Federation

Influenza A viruses from patients in the Moscow region during the outbreak of 2003 were isolated by passage in human intestinal epithelium cells (CACO-2 line). CACO-2 clinical isolates of the subtype H3N2 retained the original "human" phenotype and agglutinated human but not chicken erythrocytes. Structural properties of clinical influenza H3N2 isolates were compared and analyzed for evolutionary relationships. The following observations were made: (i) Moscow isolates showed an approximate 2-fold increase in the number of glycosylation sites of HA and NA when compared to isolates from 1968-1970; (ii) there were no amino acid exchanges in the HA receptor binding site although the viruses acquired the ability to agglutinate avian erythrocytes after passage in MDCK cells, suggesting that virus adsorption is regulated by several factors; (iii) quasispecies characterized by deletion of 66 nucleotides (22 amino acids) in the stalk region of the NA gene was dominant in naso-pharyngeal washes of all patients whereas during passaging in CACO-2 cells of isolates from different patients this deleted genotype was either stably retained as prevalent quasispecies or rapidly replaced for the one containing the full length NA gene; (iv) the NS segment was found to contain an additional positive-sense open reading frame encoding a hypothetical 25 kD polypeptide (negative strand protein -NSP) displaying transmembrane characteristics; (vii) phylogenetic analysis revealed that the NSP gene appeared at the beginning of the XIX century and in an evolutionary manner diverged in connection with the virus host range. The data suggest that increasing numbers of glycosylation sites on HA and NA and stalk shortening of NA facilitate influenza virus survival in humans. The presence of the NSP open reading frame suggests that influenza A viruses share genetic properties with ambisense RNA viruses.

7-005

### Surveillance of the evolution of epidemic human Influenza viruses during six consecutive seasons (2002/2003 to 2007/2008) in Austria

Redlberger, Monika; Popow-Kraupp, T.

Institute of Virology, Med. Univ. Vienna, Austria

**Background:** Human influenza viruses are subject to continuous antigenic drift and this phenomenon poses great problems for the recommendation of the vaccine strains in each season.

**Objectives:** (a) The antigenic and genetic characterization of influenza strains obtained from the Diagnostic Influenza Network Austria (DINOE) and from nasopharyngeal swabs from other sources, mainly hospitals; (b) to compare strains collected in Austria to the vaccine formula strains used in each season; (c) to test for the appearance of neuraminidase inhibitor-resistant influenza strains.

**Study design:** Influenza strains were collected during 6 consecutive influenza-seasons (2002/2003 to 2007/2008). Laboratory diagnosis and subtyping was done by nucleic acid amplification using Polymerase Chain Reaction (PCR) on clinical samples, followed by viral isolation in Madin-Darby canine kidney (MDCK) cells. The isolates were characterized antigenically by hemagglutination-inhibition (HI) assay with post-infection ferret antisera. The genetic characterization was performed by sequencing the HA1 portion of the HA gene. The comparison between reference and circulating strains was analyzed by the construction of phylogenetic trees. Testing for the appearance of neuraminidase inhibitor-resistant influenza strains consisted in sequencing the NA gene.

**Results:** Tracing the changes of the HA-gene by genotyping revealed that in each season, viruses started to evolve with a decreasing homology to the dominant circulating strain. These emerging strains already showed a close relationship with the dominant strain of the following influenza season. The A/H3N2 circulating strains matched the corresponding vaccine component only in season 2002/2003 and 2006/2007, whereas the circulating A/H1N1 strains matched the corresponding vaccine component in every season except in 2006/2007 and during the drift-situation in 2007/2008. As far as the Influenza viruses of Type B are concerned, a good correlation between the circulating B strains and the B vaccine component was observed in season 2004/2005 and 2006/2007. Neuraminidase inhibitor resistant strains were only detected in season 07/08 where 9 out of 92 H1N1 viruses analysed, revealed a mutation corresponding to oseltamivir resistance.

**Conclusion:** The results underscore the value of monitoring seasonal influenza strain dynamics as an instrument that can provide important and timely information on the appearance of strains with epidemiologic significance.

7-006

### Horse serum resistance of influenza B viruses

Kiseleva, Irina; Larionova, N.; Teley, L.C.P.; Isakova, I.; Voeten, J.T.M.; Rudenko, L.

Institute of Experimental Medicine RAMS, Russian Federation

One of the components present in host sera and suppressing the receptor-binding activity of influenza viruses are thermo-stable inhibitors. Sensitivity to non-specific inhibitors of the host may contribute to virus neutralization and could negatively affect the efficacy of live attenuated influenza vaccine. Some human influenza B viruses are resistant, and some are sensitive to serum thermo-stable inhibitors. To determine the molecular mechanisms of this resistance, we analyzed unique amino acid mutations in hemagglutinin (HA) which may have an influence on receptor-binding properties of these viruses. Two antigenically distinct lineages of influenza B viruses were categorized by antigenic and genetic differences of their HA, and are represented by the prototype strains, B/Victoria/2/87 and B/Yamagata/16/88. In this report, 63 influenza B viruses were characterized phenotypically and 22 of them by molecular methods. The sensitivity of influenza B viruses for thermo-stable inhibitors was measured by an HI test with normal horse serum and 0.5% chicken or 1% human type O RBCs. Amino acid sequences of influenza B viruses were collected from the international DNA databank (*GenBank*). Viruses from each of the two lineages, represented by either B/Yamagata or B/Victoria, had as many as 27 amino acid differences between their HA1 proteins and were antigenically distinct (*Rota E.A., 1992*). Most of the B viruses isolated before falling into two lineages are antigenically more related to the B/Yamagata lineage. Interestingly, it was shown that both these B viruses (isolated from 1940–1980s) and viruses of the B/Victoria lineage demonstrated strong resistance to the horse serum inhibitors. In contrast, B/Yamagata-like viruses became highly inhibitor sensitive. Twenty-two HA sequences were aligned by the computer program *Clone Manager 9 for Windows*. Among 22 influenza B viruses studied, 11 were antigenically closely related to the B/Yamagata lineage, 6 were representative of the B/Victoria lineage and 5 viruses were isolated before B viruses fell into two lineages. It was shown that viruses that demonstrated inhibitor sensitivity to normal horse serum contained four unique substitutions in HA1 (Lys-290-Met; Asn-521-Ser, Ala-682-Lys, Lys-687-Asn) compared to inhibitor resistant viruses.

**Conclusion:** Influenza B viruses antigenically closely related to the B/Yamagata lineage contain at least four unique substitutions in HA1 which may correlate with their inhibitor sensitive phenotype.

7-007

### Phylogenetic analysis of avian, human and porcine influenza A viruses isolated between 1948 and 2007

**Munster, Vincent;** de Wit, E.; Bestebroer, T.M.; Osterhaus, A.D.M.; Fouchier, R.A.M.

Department of Virology, ErasmusMC, Netherlands

The Spanish (H1N1, 1918), Asian (H2N2, 1957) and Hong Kong (H3N2, 1968) influenza pandemics killed 20-50, 1-2, and 1-2 million people globally, and ~500,000 people per year in the subsequent annual epidemics. Influenza pandemics occur when a 'new' influenza A virus is transmitted from an animal reservoir to humans and succeeds in spreading globally. Genetic mixing of avian and human viruses and/or accumulation of mutations are believed to form the basis of the generation of viruses with enhanced replication and transmission properties in humans, but little direct evidence is available. The influenza A virus that caused the H2N2 pandemic of 1957 was a reassortant virus of a contemporary human H1N1 strain with avian HA, NA and PB1 genes. The 1968 H3N2 virus was a reassortant of a human H2N2 virus with avian HA and PB1. Our aim is to unravel the molecular basis for the emergence of these pandemic influenza A viruses, by detailed comparison of previous pandemics with current pandemic threats. The Dutch National Influenza Center, housed at Erasmus MC, maintains an extraordinary collection of influenza A viruses, including human, swine and avian influenza viruses, spanning 10 years of wild bird surveillance, multiple outbreaks in poultry and mammals, and historical human influenza A viruses of the last two pandemics. We have performed full genome sequencing on a set of 120 influenza A viruses, including avian influenza viruses of all 16 HA and all 9 NA subtypes, H1N1 and H3N2 viruses isolated from swine between 1977 and 1996 and H1N1, H2N2 and H3N2 viruses isolated from humans between 1948 and 2004. Phylogenetic analysis on the nucleotide and amino acid level were used to identify avian and human progenitor gene segments for the 1957 and 1968 pandemic events. The selected progenitor gene segments will be cloned and used to study the molecular constraints for the emergence of influenza A viruses with pandemic potential.

7-008

### Antigenic drift between the A/NewCaledonia/20/99-like and A/Solomon Islands/3/2006-like H1N1 influenza viruses was due mainly to a single amino acid substitution

**Lin, Y.;** Wong, F.; Gregory, V.; Daniels, R.; Hay, A.

National Institute for Medical Research, UK

Antigenic drift between the A/NewCaledonia/20/99-like and A/Solomon Islands/3/2006-like H1N1 influenza viruses was due mainly to a single amino acid substitution. Yipu Lin, Frederick Wong, Victoria Gregory, Rod Daniels, Alan Hay, Virology Division, MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK. During the 2006-2007 influenza season, an increasing proportion of circulating H1N1 viruses was antigenically distinguishable from A/New Caledonia/20/99 and was more closely related to A/Solomon Islands/3/2006 and A/Solomon Islands/3/2006-like viruses, such as A/Fukushima/141/2006 and A/Hong Kong/2652/2006. This led the WHO to recommend that the H1N1 component of the influenza vaccine for the Northern Hemisphere 2007-2008 influenza season be changed from an A/New Caledonia/20/99-like to an A/Solomon Islands/3/2006-like virus. Sequence analysis of the HA genes of H1N1 viruses circulating in the 2006-2007 season revealed a common lysine to glutamic acid substitution (K140E) at position 140 of HA1 of A/Solomon Islands/3/2006-like viruses, relative to that of A/New Caledonia/20/99, irrespective of the phylogenetic clade/sublineage into which they fell. HA genes containing reciprocal mutations encoding single amino acid changes were generated for A/New Caledonia/20/99 (K140E) and A/Fukushima/141/2006 (E140K) and incorporated into an A/WSN/33 genetic background using reverse genetics. The antigenic properties of the chimaeric viruses produced and their respective parents were compared in hemagglutination inhibition assays with strain-specific post-infection ferret antisera. The results demonstrated that the amino acid substitution at position 140 was the principal determinant of the antigenic difference between A/New Caledonia/20/99-like and A/Solomon Islands/3/2006-like viruses.

7-009

# **Reassortant influenza B viruses with different genome compositions circulated in Germany during the past seven years**

Holdack, C.; Biere, B.; **Schweiger, B.**

Robert Koch Institute, Germany

Influenza viruses possess a segmented genome which allows an exchange of gene segments if a host is simultaneously infected with at least two different virus strains. Through this process of reassortment, influenza viruses are able to generate new virus variants with distinct gene constellations and thereby increase the genetic diversity of circulating strains. Noticeable reassortment events of influenza B viruses occur between the Yamagata- and Victoria-lineage which have evolved separately since the early 1980s. All of the eight genome segments of 86 influenza B viruses circulating in Germany between 2001 and 2007 were analysed. By phylogenetic analysis we were able to categorize each of the PB1, PB2, PA, HA, NP, NA and M segments of each isolate as being of either the Victoria or Yamagata lineage. Different reassortment patterns concerning internal genes as well as the surface antigens HA and NA were identified. The NS gene could not be classified accordingly, since it has diverged into multiple phylogenetic clusters that are independent from the Victoria- and Yamagata-categorization. However, the NS gene contributes to genetic diversity by increasing the number of different genome constellations. In addition to the genetic drift of influenza viruses, the genetic reassortment between cocirculating strains with differing genome composition is an important evolutionary mechanism which is as yet not fully understood. Our results indicate that only a few reassortment patterns of influenza B viruses have an evolutionary advantage in comparison to other genome constellations. Regarding public health issues, the exchange of segment encoding for the surface proteins HA and NA is of epidemiological interest in view of the annual WHO recommendation for influenza vaccine composition.

7-010

# **The evolution of human influenza A viruses from 1999 to 2006 - a complete genome study**

**Bragstad, K.**; Nielsen, L.; Fomsgaard, A.

Department of Virology, Statens Serum Institut, Denmark

Knowledge about the complete genome constellation of seasonal influenza A viruses from different countries is valuable for the monitoring and understanding of the evolution and migration of strains. Few complete genome sequences of influenza A viruses from Europe are publicly available at the present time and there have been few longitudinal genome studies of human influenza A viruses. We have studied the evolution of circulating human H3N2, H1N1 and H1N2 influenza A viruses from 1999 to 2006 and analysed 234 Danish human influenza A viruses as well as characterised 24 complete genomes.

H3N2 was the prevalent strain in Denmark during the study period, but H1N1 dominated the 2000-2001 season. H1N2 viruses were first observed in Denmark in 2002-2003. After years of little genetic change in the H1N1 viruses, the 2005-2006 season presented H1N1 of greater variability than before. This indicates that H1N1 viruses are evolving and that H1N1 soon is likely to be the prevalent strain again. Generally, the influenza A haemagglutinin (HA) of H3N2 viruses formed seasonal phylogenetic clusters, but different lineages co-circulating within the same season were also observed. The evolution has been stochastic, influenced by small "jumps" in genetic distance rather than constant drift, especially with the introduction of the Fujian-like viruses in 2002-2003. Also evolutionary stasis-periods were observed which might indicate well fit viruses. The evolution of H3N2 viruses have also been influenced by gene reassortments between lineages from different seasons. None of the influenza genes were influenced by strong positive selection pressure. The antigenic site B in H3N2 HA was the preferred site for genetic change during the study period probably because the site A has been masked by glycosylations. Substitutions at CTL-epitopes in the gene coding for the neuraminidase, polymerase acidic protein, matrix protein 1, non-structural protein 1 and especially the nucleoprotein were observed. The N-linked glycosylation pattern varied during the study period and the H3N2 isolates from 2004 to 2006 were highly glycosylated with ten predicted sequons in HA, the highest amount of glycosylations observed in this study period.

The present study is the first to our knowledge to characterise the evolution of complete genomes of influenza A H3N2, H1N1 and H1N2 isolates from Europe over a time period of seven years from 1999 to 2006. More precise knowledge about the circulating strains may have implications for predicting the following season strains and thereby better matching the vaccine composition.

7-011

# **Co-circulating variants of influenza A/H1N1 and influenza B viruses during the season 2007/2008 detected by rapid pyrosequencing technique**

Wedde, M.; Langnick, C.; **Schweiger, B.**

National Reference Centre for Influenza, Robert Koch Institute, Germany

Influenza A and B virus infections are major causes of morbidity and mortality worldwide. The annual vaccine recommendation necessitates a comprehensive antigenic and genetic analysis of circulating influenza viruses. Antigenic characterisation by hemagglutination inhibition (HI) requires time-consuming virus cultivation. Genetic analysis by cycle sequencing is a faster but also labour-intensive method. Here we report about a rapid pyrosequencing (PSQ) technique for detection of co-circulating variants of influenza A/H1N1 and influenza B viruses in the season 2007/08, the development of PSQ assays based on sequence data of hemagglutinin (HA) genes from influenza viruses circulating in Germany and other countries from 2006 to 2007, and the analysis of A/H1N1 sequences revealed two phylogenetic groups termed clade 1 and 2 which are further subdivided into subclade a, b and c. The two A/H1N1 clades were characterised by specific amino acid substitutions compared to the strain A/New Caledonia/20/99. PSQ assays were designed for detection of clade 1 and 2 specific substitutions. This novel method was used to analyse 274 influenza A/H1N1 viruses circulating during 2007/08. PSQ analysis revealed 97% clade 2b viruses represented by the novel vaccine strain A/Brisbane/59/07. Influenza B viruses of the Victoria lineage circulating during 2006/07 possessed no amino acid changes in their HA gene relative to B/Malaysia/2506/04. In contrast, the Yamagata lineage could be subdivided into three sublineages that were characterised by specific amino acid substitutions compared to the strain B/Florida/7/04. PSQ assays were designed for detection of the three sublineages represented by B/Celyabinsk/306/07, B/Hong Kong/864/06 and B/Florida/4/06, respectively. 250 specimens collected during the season 2007/08 were analysed using the newly established PSQ technique. In contrast to the homogenous A/H1N1 viruses, PSQ analysis of influenza B viruses revealed a much more heterogeneous spread caused by co-circulating Yamagata sublineages. About 36% were characterised as B/Celyabinsk/306/07-, 20% as B/Hong Kong/864/06- and 44% as B/Florida/4/06-like viruses, respectively. In conclusion, PSQ is a valuable technique for surveillance and fast genetic analysis of co-circulating influenza virus variants. Moreover, it provides precocious information about circulating variants essential for an optimal annual vaccine composition.



7-012

# **Whole-genome sequencing of influenza A and its usefulness in surveillance of circulating seasonal influenza**

**Galiano, M.C.<sup>1</sup>**; Baillie, G.<sup>2</sup>; Quail, M.<sup>2</sup>; Lackenby, A.<sup>1</sup>; Democratis, J.<sup>1</sup>; Xiang, Z.<sup>3</sup>; Hou, T.<sup>3</sup>; Ellis, J.<sup>1</sup>; Hay, A.J.<sup>3</sup>; Zambon, M.C.<sup>1</sup>; Daniels, R.S.<sup>3</sup>

<sup>1</sup>Influenza Laboratory, Virus Reference Division, Centre for Infections, HPA Colindale, London, UK; <sup>2</sup>The Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge, UK; <sup>3</sup>Virology Division, MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London, UK

Analysis of all eight segments comprising the whole influenza A virus genome is increasingly used to supplement influenza virus strain surveillance. In particular, it helps to provide a better understanding of mutational events, segment co-segregation and reassortment contributing to human influenza A virus evolution. Achieving high throughput whole genome sequencing of human clinical isolates and directly from human clinical material requires optimisation of segment amplification and sequencing techniques to ensure a rapid response capability, as would be required in the event of the emergence of novel human or pandemic virus strains.

We have evaluated the potential of highly automated approaches, as pioneered in human genome sequencing, for influenza A genomic sequencing, using library generation with concatemer formation. This involves ligating a library of PCR products covering a complete virus genome, sonication of concatemeric products to yield fragments of ~1Kb, cloning and shot-gun sequencing the fragments. Such an approach has the potential to generate a truly high throughput method that will work with any known or unknown influenza virus, which is highly automatable, unlike conventional primer walking which is specific for known viral subtypes. The concatemeric approach reproducibly yields significant viral sequence coverage but often small regions of the virus are underrepresented and further follow-up finishing is needed. However, this approach could work for scanning larger viral populations without sequencing to completion. In contrast, more traditional primer walking techniques reliably generate whole segment sequence coverage, but require more reagents, more manual handling and a starting knowledge of virus subtype.

The usefulness of whole-genome sequencing can be shown in different biological situations. The emergence of oseltamivir resistant A/Solomon Islands/03/2006-like H1N1 viruses in winter 2007-2008, in the absence of drug pressure was an unexpected event. Viruses bearing the NA mutation His 274 Tyr (H274Y) have occurred at low frequency (< 1% in unselected surveillance) since the introduction of oseltamivir, but have previously not been readily transmissible between infected individuals. Resistant H1N1 viruses circulating in 2007-08 have transmitted readily

between individuals, suggesting that compensatory mutations in the viral genome have allowed influenza A strains carrying H274Y to overcome previously observed growth and transmission disadvantages. We discuss the results from whole genome sequence analysis of over 34 H1N1 reference and circulating strains, generated through the UK influenza sequencing pipeline project and evaluate the possible contribution of co-segregating compensatory mutations in virus polymerases.

7-014

#### Genetic and antigenetic structure of influenza virus A H1N1 strains isolated in Russia in 2005-2008 epidemic seasons

**Pisareva, M.;** Kononova, N.; Zadonskaya, A.; Komissarov, A.; Yaglovskaya, I.; Erokin, M.; Grudin, M.; Kiselev, O.

*Research Institute of Influenza, Russian Federation*

Influenza epidemics in Russia occur virtually every year, being determined by continuous variability of modern influenza A and B viruses. Such an epidemic situation is complicated by co-circulating influenza viruses of not only different types and subtypes but different antigenic variants.

Analyses of the viral population of the last epidemic seasons showed a substantial increase of H1N1 viruses. Thus during the 2005-2006 epidemic season, the number of viruses of this subtype did not exceed 12% from all strains isolated in Russia. During the following epidemic seasons of 2006-2007 and 2007-2008 it reached 36.9% and 78.4% correspondingly. Every year about 500 influenza A and B virus strains are isolated in Russia by the Research Institute of Influenza and its basic laboratories.

According to antigenic and molecular analyses, the activation of H1N1 was associated with the appearance of a new antigenic variety of viruses. Russian isolates of the 2006-2007 epidemic season differed from the vaccine strain A/New Caledonia/20/99 in 9-12 amino acid positions. However Russian 2006-2007 isolates (except A/Khabarovsk/515/07) had no essential substitutions (K73R, V128T, K140E) discriminating new reference strain A/Solomon Islands/3/06 but possessed additional substitutions S36N, E169K, N183T, R188S and A189T. So the majority of H1N1 viruses circulating in Russia in 2006-2007 formed the cluster of strains that by their antigenic and genetic features differed from both old and new vaccine strains.

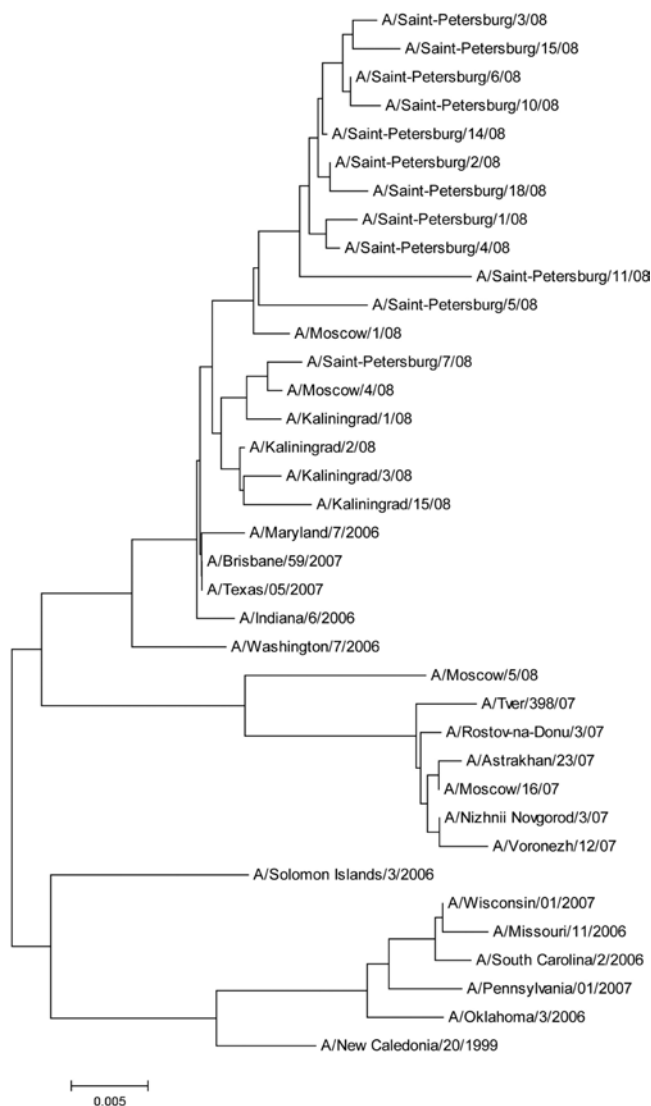
Epidemic events in Russia in 2007-2008 were characterized by more intensive circulation of influenza A H1N1 viruses. Strains of this epidemic season were antigenically and genetically closely related to the last reference strain A/Brisbane/59/07 (See fig.1). Interestingly most Russian isolates of 2007-2008 are not a direct evolutionary continuation of 2005-2007 viruses and could be separated out in an independent group with a certain set of

amino acid substitutions in HA1. Comparative analysis of these strains with vaccine and previous Russian strains showed the differences in 6 amino acids (D35S, T82K, Y94H, K140E, V165A, R188K) from A/New Caledonia/20/99, 5 amino acids (D35S, S73K, T128V, K145R, R188K) from A/Solomon Islands/3/06, 1 amino acid (D, G, N, V186I) from A/Brisbane/59/07 and 8 amino acids (D35S, N36S, K140E, K145R, K169E, T183N, S188K, T189A) from Russian strains isolated in 2006-2007. It should be noted that all 2008 Russian isolates have glutamic acid residue instead of lysine at position 140 in Ca antigenic site. This charge-changeable substitution essentially influences viral antigenic features confirmed by antigenic analysis.

Evolutionary changes affected not only HA but the second surface protein NA as well. Russian 2008 strains possess all substitutions characteristic for A/Brisbane/59/07 and differ from A/New Caledonia/20/99 and A/Solomon Islands/3/06 in 7-9 residues of NA. The most significant amino acid changes are registered at the residues E214G, R222Q, G249K, T287I, K329E, D344N and G354D of the last Russian isolates. All analyzed 2008 strains have lysine instead of arginine or glycine at residue 249 indicating possible zanamivir resistance of new Russian isolates (L.V. Gubareva *et al.*, 1996). Practically all 2008 isolates from Saint Petersburg have tyrosine instead of histidine at residue 275. This substitution leads to oseltamivir resistance (L.V. Gubareva *et al.*, 2001). Molecular analysis confirmed by biological analysis revealed 55.6% oseltamivir-resistant strains among analyzed Russian isolates in 2008.

Long-term monitoring showed that the accumulation of point mutations in HA and NA genes led to the shift of the epidemically urgent influenza A virus variant. Influenza viruses often evolve in the so-called "silent way" when mutations in the HA gene do not manifest until their accumulation does not lead to the appearance of the virus with antigenic and genetic features optimal for epidemic prevalence. Such an evolutionary route could be traced in appearance of the new antigenic variant A/Brisbane/59/07.

Fig.1. Phylogenetic tree of HA gene of influenza A virus H1N1 strains isolated in 2006-2008



7-015



## Neuraminidase substrate specificity in avian-human reassortants

**Shtyrya, Y.A.<sup>1</sup>; Mochalova, L.V.<sup>2</sup>; Rudneva, I.A.<sup>3</sup>; Shilov, A.<sup>3</sup>; Kaverin, N.V.<sup>3</sup>; Bovin, N.V.<sup>1</sup>**

<sup>1</sup>Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Russian Federation; <sup>2</sup>Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Russian Federation; <sup>3</sup>Ivanovsky Institute of Virology, Russian Academy of Medical Sciences, Russian Federation

Reassortation (i.e. exchange of RNA segments between two different viruses simultaneously infecting the same cell) is considered to be the main mechanism for forming different types of influenza viruses in nature. Commonly, reassortants have hemagglutinin (HA) and neuraminidase (NA) from different parent viruses, which leads to functional imbalance in newly formed variants. Only reassortant viruses capable of restoring HA/NA balance maintain in viral population. The analysis of natural avian influenza viruses revealed that some combinations of HA and NA antigenic subtypes occurred frequently, while others were rare, or not detected at all (1); the reason for this selectivity still remains unknown. The procedure of obtaining reassortant viruses with desired gene combinations is well known and is rather simple, allowing for example to construct viruses with avian HA and human NA.

This work was focused on understanding mechanisms which lead to restoration of functional balance in case of human-avian reassortant viruses, especially, changes in the influenza virus NA substrate specificity.

To this end, three reassortant viruses, R8/Dk-Rai, R2 and RCB (of H3N2, H3N1 and H4N1 subtypes consequently) and their passage variants (R8/Dk-RAiXII, R2-XXI and RCB-XXI) adapted for growth on embryonated chicken eggs, which acquired different changes in the NA gene during their adaptation, were selected from the pool of previously obtained reassortants (2-4). All these passage viruses had changes in HA near the receptor binding site, leading to a decrease in their affinity to carbohydrate receptor (2-4).

The study of influenza virus NA substrate specificity was performed with seven BODIPY-labelled sialyloligosaccharides (Table 1) differing by: 1) the type of linkage between the Neu5Ac and Gal residues, 2) the presence of an N-acetamide group at position 2 of the Gal residue, 3) core type (1-3 or 1-4), and 4) the presence of fucose at the GlcNAc residue. The method has been used before for investigation of oligosaccharide specificity of H1N1 neuraminidases (5, 6).

Table 1  
Structure of BODIPY-labelled sialyloligosaccharides

Oligosaccharide	Abbreviation
Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-4Glc	3'SiaLac
Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-4GlcNAc	3'SiaLacNAc
Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-3GlcNAc	SiaLe <sup>c</sup>
Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-3(Fuc $\alpha$ 1-4)GlcNAc	SiaLe <sup>A</sup>
Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc	SiaLe <sup>x</sup>
Neu5Ac $\alpha$ 2-6Gal $\beta$ 1-4Glc	6'SiaLac
Neu5Ac $\alpha$ 2-6Gal $\beta$ 1-4GlcNAc	6'SiaLacNAc

The NA of the viruses R8/Dk-Rai and R8/Dk-RAiXII (H<sub>3</sub>N<sub>2</sub>) differed only by a single amino acid in the NA gene (Glu83Gly). This amino acid is situated quite far from the NA active site and it is not surprising that it caused only minor changes in the NA substrate specificity profile. The decrease in activity towards SiaLe<sup>A</sup> from 2.8 to 2 when compared to the activity towards SiaLe<sup>c</sup>, and the decrease in activity for SiaLe<sup>x</sup> from 3.6 to 2.5 when compared to 3'SiaLacNAc are the main changes that occurred during adaptation of this reassortant virus.

The NA of adaptant R2-XXI (H<sub>3</sub>N<sub>1</sub>) differed from R2 (H<sub>3</sub>N<sub>1</sub>) by two amino acid residues (Asp79Val and Ser366Asn), the first of them being located in the same region as in the R8/Dk-Rai - R8/Dk-RAiXII pair of viruses, and is likely to cause only minor changes in the NA substrate specificity profile. As for the second change – this is located in close proximity to the hemoadsorption site and is likely to cause a major difference between specificity profiles. It is also worth mentioning that in this case, there was an occurrence of reversion from the amino acid type typical for NAs of human viruses to the one which is typical for avian ones. The most remarkable difference between specificity profiles includes changes in activity ratio for SiaLe<sup>c</sup>/SiaLe<sup>A</sup> from 3.6 to 4.3, for 3'SiaLacNAc/SiaLe<sup>x</sup> from 1.6 to 2.7, as well as a change in activity ratio towards 3'SiaLacNAc/6'SiaLacNAc from 4.6 to 5.5 (for R2 and R2-XXI respectively).

In the process of adapting the RCB reassortant, its NA saw only one change (Leu206Ile). It is quite remarkable that this minor synonymous change had truly dramatic effects on the specificity profile of RCB-XXI the passage variant, in particular the ability to discriminate between  $\alpha$ 2-3/ $\alpha$ 2-6 SiaLac increased from 3.9 for reassortant to 9.6 for passage variant. The same tendency is observed for  $\alpha$ 2-3/ $\alpha$ 2-6 SiaLacNAc where the ratio change is from 5.6 to 7.3. The NA of the passage variant also has a decreased value for ratio 3'SiaLacNAc/SiaLe<sup>c</sup> from 3.2 (for reassortant) to 1.6 for passage variant.

The only explanation for such a dramatic specificity change after only synonymous substitution is that this amino acid is located in one of the  $\beta$ -strands in the region of NA which is strictly conservative between the NA of different origin and subtypes. As for such a conservative region, even the minor change in structure of amino acid may cause major effects on the stability of this part of the polypeptide chain which may

affect the enzymatic activity.

All amino acid changes that have occurred in passage variants caused changes in the substrate specificity profile. This indicates the need for a thorough investigation of the role of different amino acids in substrate specificity and activity, especially in the application of changes that occur in the course of restoring functional HA-NA balance for naturally occurring reassortants. All amino acid changes in the described cases caused a change from the profile common for NAs of human viruses, to profiles which are closer to those established for NAs of poultry and avian viruses.

We suggest three modes of functional HA-NA balance restoration. The first one is represented by R8/Dk-Rai and R8/Dk-RAiXII viruses, where major changes have occurred in the functional activity of HA and are accompanied by minor changes in NA specificity. In the second case (RCB1 and RCB1 XXI viruses) on the contrary, most dramatic changes have occurred in the NA specificity profile. And in the third case (R2 and R2 XXI pair), changes occurred in the HA functional activity as well as the NA substrate specificity.

Summing up, the changes in human NA and avian HA during passaging of reassortant viruses take place in concert with respect to the oligosaccharide specificity of HA and NA. Apparently, one of the factors determining a functional balance between the HA and NA in the viable virus, is the compatibility of the receptor-binding specificity of HA and substrate specificity of NA.

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7-016

### Evolutionary interactions between haemagglutinin and neuraminidase in avian influenza

**Ward, M.;** Bollback, J.P.; Lycett, S.J.; Leigh Brown, A.J.

University of Edinburgh, UK

Highly pathogenic avian influenza viruses of haemagglutinin (HA) subtypes H5 and H7 present an epidemiological and economic threat on a global scale. The HA gene, which encodes the major surface antigen of influenza, is thought to be under selective pressure to evolve to evade the host immune response. Evidence for positive selection in HA in human viruses has been

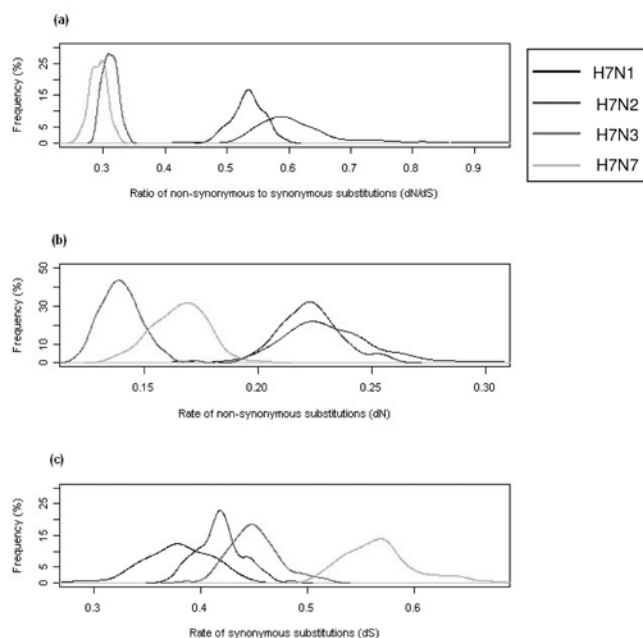
provided in terms of a significantly high ratio of non-synonymous to synonymous mutations ( $d_N/d_S$ ) [e.g. Bush *et al.* 1999]. Reassortment between HA and neuraminidase (NA), the other major antigenic influenza gene, produces novel combinations of subtypes to which the majority of a host population will be naïve. However, few studies have considered how the genetic interactions between different segments affect their evolution [Rambaut *et al.* 2008]. We investigate how the association of H7 subtype HA with different NA subtypes influences the evolutionary rate of that segment. Unlike previous studies, a Bayesian approach was adopted for estimating parameters, topologies and mutational histories.

We downloaded all available H7 subtype HA avian influenza sequences from the NCBI database [http://www.ncbi.nlm.nih.gov] and grouped them according to their NA subtype. After excluding NA subtypes for which there were few sequences, a dataset comprising of 253 sequences of subtypes H7N1, H7N2, H7N3 and H7N7 was aligned. Samples of parameters and topologies under the GTR +  $\Gamma$  model of DNA substitution were obtained using MrBayes [Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003]. The MrBayes consensus tree revealed phylogenetically distinct clades of North American and Eurasian viruses, confirming an earlier report [Banks *et al.* 2000]. The stochastic mutational mapping method of Nielsen (2001, 2002) was used to estimate synonymous and non-synonymous evolutionary rates ( $d_S$  and  $d_N$  respectively) for each lineage. This approach is particularly useful for a number of reasons. Firstly, mutational mapping allows  $d_N$  and  $d_S$  to be estimated independently, whereas traditional maximum likelihood methods estimate the composite  $d_N/d_S$  ratio (e.g. Chen and Holmes 2006). Secondly, the mutational mapping approach naturally accommodates uncertainty in the topology and the parameters of the substitution model by treating mutations as missing data and sampling from the posterior distribution. Lastly, mutational mapping possesses advantages over the classical method of maximum parsimony, which excludes many possible non-parsimonious mappings that have a high probability of occurrence. We used SIMMAP [Bollback 2006] to sample mutational mappings (i.e. samples from the posterior distribution) on the parameters and topologies from MrBayes.

We found substantial variation in  $d_N/d_S$  ratios of H7 HA sequences across different NA subtypes (fig. a). Our rankings concur with earlier observations that H7N1 and H7N2 HA sequences exhibited higher  $d_N/d_S$  ratios than H7N3 and H7N7 HA sequences (Chen and Holmes 2006). Using the mutational mapping approach we are able to explain this pattern in terms of dramatic differences in the underlying evolutionary behaviour of H7 HA associated with different NA subtypes. Rates of non-synonymous substitution were significantly higher for H7N1 and H7N2 than for H7N3 and H7N7 (fig. b). A total of 7 amino acid sites of the HA gene appear to be under significant positive selection, contributing to this elevated rate. Whilst some sites matched, or were proximal to, known glycosylation sites, others have not been previously reported as having functional or antigenic importance. We

conclude that reassortment exposes HA sequences to significant changes in selective forces through direct or indirect epistasis with NA.

\* email: so677954@sms.ed.ac.uk



The ratio of non-synonymous to synonymous substitutions ( $d_N/d_S$ ) in HA sequences was calculated for each mutational mapping. The distribution of  $d_N/d_S$  ratios across mutational mappings is shown in fig (a), with the distribution for H7N1 shown in black, H7N2 in blue, H7N3 in red and H7N7 in green. Figures (b) and (c) show the distribution of non-synonymous substitution rates ( $d_N$ ) and synonymous substitution rates ( $d_S$ ) respectively across mutational mappings.

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7-017

### Molecular epidemiology of A/H3N2 and A/H1N1 influenza virus during a single epidemic season in the United States

**Nelson, M.I.<sup>1</sup>; Viboud, C.<sup>2</sup>; Simonsen, L.<sup>3</sup>; Miller, M.A.<sup>2</sup>; Edelman, L.<sup>4</sup>; Halpin, R.<sup>5</sup>; Spiro, D.J.<sup>5</sup>; Holmes, E.C.<sup>1</sup>**

<sup>1</sup>The Center for Infectious Disease Dynamics, The Pennsylvania State University, USA; <sup>2</sup>The Fogarty International Center, The National Institutes of Health, USA; <sup>3</sup>School of Public Health and Human Services, The George Washington University, USA; <sup>4</sup>Surveillance Data Inc., USA; <sup>5</sup>The J. Craig Venter Institute, USA

To determine the spatial and temporal dynamics of influenza A virus during a single epidemic season, we examined whole-genome sequences of 284 A/H1N1 and 69 A/H3N2 viruses collected across continental United States during the 2006-2007 epidemic, representing the largest study of its kind undertaken to date. A phylogenetic analysis revealed that multiple antigenically distinct clades of both A/H1N1 and A/H3N2 entered and co-circulated during this season, with no clear pattern of spatial spread. Multiple clades co-circulated in most localities, even those that are distant from major metropolitan areas. In addition, co-circulating clades frequently exchanged genome segments through reassortment, including a minor clade of A/H3N2 viruses that appears to have re-acquired sensitivity to adamantane drugs, as well as a minor A/H1N1 clade that later became globally dominant. Overall, the co-circulation of multiple clades during the 2006-2007 epidemic season, produces patterns of spatial spread that are far more complex than previously thought, and supports a key role for migration in shaping the epidemiological dynamics of influenza A virus.

In particular, eight distinct clades of A/H1N1 influenza virus are evident on the phylogenetic tree constructed for each of the eight genome segments (PB2, PB1, PA, HA, NP, NA, M, and NS). Each of these clades is likely to represent a separate introduction of the virus into the United States, and all eight of these clades co-circulated over a wide spatial-temporal scale across the U.S. Multiple clades were observed among all localities from which >1 isolate was collected, and genetic diversity was extensive both in major metropolitan areas and more remote areas. However, in contrast to a simplified spatial model in which a single lineage spreads in a unidirectional manner, we observed no strong spatial signal among the co-circulating clades, even when the major clade (denoted 'clade A') containing the most isolates (175/284,

61.6%) was studied in isolation.

These eight A/H1N1 clades varied phenotypically, including at least three different antigenic types, as well as both adamantane resistant and sensitive viruses (Figure 1a). Based on a phylogenetic relation to antigenically distinct vaccine reference strains and amino acid differences in key antigenic sites, clades A, B, C, D, and E appear to be A/New Caledonia/20/1999-like in antigenicity, clade F (and perhaps clade G) appear to be antigenically A/Brisbane/59/2007-like, and clade H appears to be A/Solomon Islands/3/2006-like. Furthermore, clade G exhibited the S31N mutation in the M2 gene associated with resistance to adamantane drugs, while the other seven clades remained adamantane sensitive.

Although fewer A/H3N2 influenza viruses (n = 69) were available for study due to the dominance of the A/H1N1 subtype during this epidemic, significant phylogenetic diversity is also evident for all eight genome segments. In addition to a major clade of A/H3N2 viruses (also denoted 'clade A'), a distinct minor clade (clade B) and four singleton viruses (S1, S2, S3, and S4) all co-circulated (Figure 1b). These clades exhibited extensive phenotypic variation, representing at least two known antigenic types (and possibly two new antigenic types), and also included both adamantane sensitive and resistant viruses. Clade A was antigenically A/Brisbane/10/2007-like, singleton isolates S1, S2, and S3 were A/Wisconsin/67/2005-like, and clade B and isolate S4 were both of unknown antigenicity. While clade A and isolates S1, S2, and S3 were resistant to adamantane, clade B and isolate S4 were adamantane sensitive.

Reassortment was important in generating the viral diversity observed in both the A/H3N2 and A/H1N1 subtypes. Intra-subtype reassortment among A/H1N1 influenza virus produced the antigenically novel clade F viruses, which contain four genome segments (PB1, NP, M, and NS) that relate to the majority of other A/H1N1 viruses from this epidemic (clades A, B, C, and D) and four segments (PB2, PA, HA, and NA) that are highly divergent, including the key surface antigens (HA and NA). Among A/H3N2 influenza viruses, clade B and isolate S4 both appear to have re-acquired sensitivity to adamantane drugs through a reassortment event involving the M and PB1 segments. Clades A and B also appear to have underwent another reassortment event involving the NP segment. Lastly, the varying phylogenetic positions of the S4 isolate across the genome suggest that this singleton virus also resulted from multi-segment reassortment.

In sum, the co-circulation of multiple subtypes and lineages complicates any analysis of spatial dynamics. Rather than a single viral lineage spreading across the continent, multiple antigenically variant lineages of both A/H3N2 and A/H1N1 influenza virus are separately introduced and co-circulate contemporaneously, allowing for reassortment within subtypes that can produce novel phenotypes. The role of reassortment in the reacquisition of adamantane sensitivity by clades of A/H3N2 influenza virus was particularly notable. By sampling in both metropolitan and relatively isolated areas, our study revealed that extensive viral diversity, including multiple antigenically distinguishable lineages

from both viral subtypes, disseminates widely across the entire United States during a single epidemic, even into relatively remote areas. Further sequencing of global influenza viruses, particularly in Asia, is needed to trace the geographic origins of the influenza viruses that enter the United States at the start of each epidemic. Importantly, it is also possible that the 2006-2007 U.S. epidemic was particularly difficult to analyze with respect to spatial-temporal patterns due to the unusual complexity of this epidemic's evolutionary dynamics and the extent of genetic and antigenic diversity. Hence, repeating this sampling effort during an influenza season in which a single lineage dominates, substantially increasing the number of sequences, and minimizing any geographical biases could increase the likelihood of obtaining a strong spatial signal.

Figure 1. (a) Phylogenetic relationships of the HA gene of 69 A/H3N2 influenza A viruses sampled from the United States during the 2006-2007 influenza season and 104 background global A/H3N2 influenza viruses sampled from 2003-2006. Clades of related viral isolates from the 2006-2007 U.S. epidemic are denoted by colored rectangles: major clade (A), minor clade (B), and four singleton isolates (S1, S2, S3, and S4). The antigenicity (A/Wisconsin/67/2005(WISC05)-like, A/Brisbane/10/2007(BRIS07)-like, or unknown) and sensitivity to adamantane is listed in parentheses for each clade. (b) Phylogenetic relationships of the HA gene of 100 influenza A viruses of the H1N1 subtype sub-sampled from all eight clades (A-H) that co-circulated in the United States during the 2006-2007 influenza season and 67 global isolates from 2001-2006. The antigenicity (A/New Caledonia/20/1999(NC99)-like, A/Solomon Islands/3/2006(SI06)-like, A/Brisbane/59/2007(BRIS07)-like, or unknown) and sensitivity to adamantane is listed in parentheses for each clade. Both trees are estimated using an ML method and mid-point rooted for purposes of clarity only. Bootstrap values (>70%) are shown for key nodes, and all horizontal branch lengths are drawn to scale.

7-018

# **Expansion of BioHealthBase to support management and analysis of data from National Institutes of Health-funded Centers of Excellence in Influenza Research and Surveillance**

**Macken, Catherine<sup>1</sup>**; Squires, B.<sup>2</sup>; Baumgarth, N.<sup>3</sup>; Dietrich, J.<sup>4</sup>; Klem, E.B.<sup>4</sup>; Scheuermann, R.H.<sup>2</sup>

<sup>1</sup>Los Alamos National Laboratory, USA; <sup>2</sup>University of Texas Southwestern Medical Center, Dallas, Texas, USA; <sup>3</sup>University of California at Davis, Davis, California, USA; <sup>4</sup>Northrop Grumman IT, Rockville, Maryland, USA

BioHealthBase is one of eight National Institutes of Health (USA)-funded Bioinformatics Resource Centers (BRC), contracted in 2004 to provide support for research on pathogens of special interest. One of the five pathogens of special interest covered by BioHealthBase is influenza virus. In 2007, BioHealthBase expanded beyond its original mandate to begin supporting the newly NIH-funded Centers of Excellence in Influenza Research and Surveillance (CEIRS). As the CEIRS generate data from surveillance and research activities, these data are being saved in the BioHealthBase BRC.

CEIRS surveillance is carried out primarily in Eastern Europe, Asia and North America. Resultant data will be web-accessible at BioHealthBase, for download and for statistical analysis. Selected CEIRS experiment data will be stored in BioHealthBase data structures that facilitate collation and comparison of data from experiments across different Centers.

This compilation of CEIRS data into a centralized database will help researchers in multiple ways. Importantly, it will enhance the ability of influenza researchers to aggregate related data from different sources, thereby increasing the power of subsequent statistical analysis. It will also facilitate construction of datasets for meta-analyses.

Our presentation will describe initial BioHealthBase capabilities for web-driven analysis of CEIRS surveillance data, and web-driven assimilation of CEIRS experiment data. Through examples, we will demonstrate the potential for substantially enhanced and accelerated influenza viral research as a result of storing data from the disparate CEIRS projects in a single, comprehensive resource. All services of BioHealthBase are freely available to all members of the influenza research community.

## 8 ANIMAL INFLUENZA & ECOLOGY

8-001

### Molecular testing strategies during UK avian influenza (AI) outbreaks in 2007 and 2008: The role of AI RealTime PCRs

**Slomka, Marek**<sup>1</sup>; Hesterberg, U.<sup>2</sup>; Pavlidis, T.<sup>2</sup>; Londt, B.Z.<sup>2</sup>; Manvell, R.<sup>2</sup>; Banks, J.<sup>2</sup>; Irvine, R.<sup>2</sup>; Brown, I.H.<sup>2</sup>; Alexander, D.J.<sup>2</sup>

<sup>1</sup>Veterinary Laboratories Agency (VLA), UK; <sup>2</sup>VLA, UK

**Introduction:** Three avian influenza (AI) poultry outbreaks occurred in the UK in 2007: two were H5N1 highly pathogenic (HP)AI and one was H7N2 low pathogenicity (LP)AI. AI RealTime PCR testing strategies were appropriately gauged to these distinct outbreak scenarios. These poultry outbreaks and subsequent H5N1 HPAI cases in Mute Swans in early 2008 demonstrate how RealTime PCR provides rapid and accurate information for the management of AI.

**Materials and Methods:** Clinical specimens were collected from poultry for: (i) Disease diagnosis at the originating infected premises (IP) and (ii) screening within the surrounding Protection and Surveillance zones. RNA was extracted robotically from specimens using a Qiagen BioRobot. Validated AI RealTime PCRs for M gene, H5, H7 and N1 genes plus standard methods for AI isolation and serology were conducted as described (1, 2). For molecular phylogeny of the H5N1 HP and H7N2 LP AI isolates, haemagglutinin gene sequence data (obtained by BigDye sequencing, Applied Biosystems) was analysed by the PHYLIP molecular phylogeny software package (3).

**Results:** The platforms of M, H5, H7 and N1 gene RealTime PCRs were used in unison to diagnose the index cases and provide evidence of either limited or no spread of AI. This led to the prompt establishment of Protection, Surveillance and Buffer Zones in accordance with EU AI policy. RealTime PCR data will be presented to summarise the following: a) Disease diagnosis: Numbers of AI positives at the originating infected premises (IPs). b) Poultry screening: Extensive testing was conducted in the Protection and Surveillance Zones. These included epidemiologically identified dangerous contact (DC) premises. c) Key decisions to change testing from M gene RealTime PCR to respective H5/H7 RealTime PCRs will be explained. d) Differences in testing strategy between HP and LP AI outbreaks will be emphasised. e) Environmental specimens from the H5N1 HPAI turkey outbreak in November 2007 also yielded positive results by H5 RealTime PCR. There was a clear correlation between H5 positive RealTime PCR results in the environment and prevalence of H5N1 HPAI infection in turkeys within a given epidemiological unit. f) Outcomes from accompanying wild bird surveillance by AI RealTime PCR will be noted. g) An update will be provided describing use of AI RealTime PCRs in the H5N1 HPAI wild bird incident at Abbotsbury, Dorset in early 2008. h) Molecular epidemiology: Phylogenetic analyses

of these UK H5N1 HPAI isolates will also be presented in relation to other clade 2.2 H5N1 HPAI isolates. The UK H7N2 LP AI isolate (2007) will be similarly compared to other recent European H7 isolates from poultry and wild birds.

**Discussion and Conclusions:** Virus isolation remains the gold standard for identifying and reporting an index case from any new AI outbreak. However, widespread use of validated AI RealTime PCRs in EU labs has demonstrated clear advantages since the H5N1 HPAI incursions into Europe in 2006. Swift and effective control of LP and HPAI UK poultry outbreaks in 2007 further illustrated the benefits of rapid, sensitive and specific testing with high sample throughput. The etiology and epidemiological details of these AI outbreaks necessitated use of appropriate RealTime PCR testing strategies.

**Acknowledgements:** The authors gratefully acknowledge the contribution of all scientific and veterinary colleagues in diagnosing and controlling these UK H5N1 HPAI and H7N2 LP AI outbreaks.

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8-002

### Seroprevalence to influenza virus strains in several non-human primate species

**Weinkauf, J.L.**<sup>1</sup>; Albrecht, R.A.<sup>1</sup>; Calle, P.P.<sup>2</sup>; Karesh, W.B.<sup>2</sup>; García-Sastre, A.<sup>1</sup>

<sup>1</sup>Department of Microbiology Mount Sinai School of Medicine, USA; <sup>2</sup>Wildlife Conservation Society, USA

Influenza infection is a significant public health problem; H1N1 and H3N2 influenza viruses are responsible for killing some 30,000 people per year in the US alone, despite widespread annual vaccination. In addition, subtypes of avian influenza viruses have infected humans, demonstrating potential for a novel human influenza virus pandemic strain. Extensive serological surveys are important for surveillance and for understanding inter-species transmission, however they present social and political difficulties. There are currently only limited published reports of influenza serological surveys in humans as well as other species. Interestingly, the susceptibility of non-human primates

to infections with naturally occurring influenza virus strains is not well understood.

To gain insight on this, 122 serum samples from eight species of non-human primates that were provided by the Wildlife Conservation Society, Bronx, New York, were tested for previous exposure and seroconversion to representative H1, H3, H5, and H7 strains of influenza viruses using a hemagglutination inhibition assay. A high seroprevalence to H1 (61%) and H3 (94%) strains was found. We also observed a high seroprevalence to H5 (51%) and H7 (20%) strains. Interestingly, two species, *Lemur catta* (lemur) and *Papio hamadryas* (baboon) demonstrated complete seronegativity to H5 and H7 strains. This suggests either infection without seroconversion, a complete resistance of these species to infection with these subtypes, different historic exposure to these strains, or differential exposure risks in these species or their exhibits. These findings are being confirmed by western blot assays to demonstrate the presence of anti-influenza virus antibodies in the primate sera.

Our results suggest that several species of non-human primates have been infected with H1, H3, H5 and H7 subtypes of influenza viruses, but the ability of these viruses to replicate in non-human primates and to induce disease is at present unknown. The observed seroconversion could result from natural transmission of influenza viruses from human or avian species to non-human primates that were housed in open-air exhibits. In addition, it is possible that some species of non-human primates may harbour novel non-human, primate-specific strains of influenza viruses.

at the time of migration is associated with increased susceptibility of migratory birds to viral infection, and in particular to infection with highly pathogenic avian influenza (HPAI) H5N1 virus. An increased susceptibility to infection with HPAI H5N1 virus may result in increased viral excretion or increased severity of disease, which may have important consequences for the long distance spread of the virus by migratory birds. Because migratory changes, including the increase in plasma concentration of corticosterone still occur in captive red knots (*Calidris canutus*), independently of the actual migratory journey, this species is well-suited for laboratory studies of migration physiology. Therefore, we used this species as a migratory bird model to determine whether or not birds were more susceptible to infection with the HPAI H5N1 virus during spring migration in relation to the observed increase in plasma concentration of corticosterone. Groups of five to six red knots were inoculated before, during and after the period of spring migration intra-tracheally and intra-oesophageally with  $10^6$  TCID<sub>50</sub> of A/turkey/Turkey/1/2005. Blood samples were collected before inoculation to measure plasma concentration of corticosterone and to determine correlates of constitutive immunity prior to infection. The birds were observed for clinical signs, and pharyngeal and cloacal swabs collected daily until quantitative PCR demonstrated that they stopped excreting. Birds showing severe clinical signs or birds which stopped excreting for 2 consecutive days were euthanized and organs were collected for virological, histopathological and immunohistochemical examinations. Red knots excreted virus from the pharynx for up to 5 days post-inoculation (dpi) and viral titers peaked at up to  $10^{5.2}$  TCID<sub>50</sub>/ml. No virus was recovered from cloacal swabs of any birds. Two birds infected before migration, and one bird infected at the time of migration developed neurological signs between 5 and 6 dpi, associated with encephalitis. Although plasma concentration of corticosterone did not differ significantly between the three groups of birds, pharyngeal excretion of HPAI H5N1 virus was positively correlated with plasma concentration of corticosterone. These results demonstrate that red knots with higher plasma concentrations of corticosterone excrete more HPAI H5N1 virus. If this is a more general phenomenon, the physiological changes at the time of migration might favour the long-distance dispersal of viruses by migratory birds.

8-004



#### Pharyngeal excretion of highly pathogenic avian influenza H5N1 virus correlates with plasma concentration of corticosterone in a migratory bird species

**Reperant, L.A.<sup>1</sup>; van de Bildt, M.W.G.<sup>2</sup>; van Amerongen, G.<sup>2</sup>; Buehler, D.M.<sup>3</sup>; Osterhaus, A.D.<sup>2</sup>; Dobson, A.P.<sup>4</sup>; Piersma, T.<sup>3</sup>; Kuiken, T.<sup>2</sup>**

<sup>1</sup>Princeton University and Erasmus Medical Centre, Netherlands; <sup>2</sup>Erasmus Medical Centre, Netherlands; <sup>3</sup>University of Groningen, Netherlands; <sup>4</sup>Princeton University, USA

Higher plasma concentration of corticosterone is reported in a number of migratory bird species just prior to or at the time of migration, and this is thought to contribute to preparation for long-distance flights. Because corticosterone also interferes with the immune system, migratory birds are believed to be more susceptible to infections during migration. Reactivation of latent infections with *Borrelia burgdorferi* has been experimentally evidenced in songbirds as a result of migratory restlessness. It is unknown whether higher plasma concentration of corticosterone

8-005

### Ecology and evolution of avian influenza viruses in the Camargue (southern France)

Lebarbanchon, C.<sup>1</sup>; Chang, C.M.<sup>2</sup>; Grandhomme, V.<sup>2</sup>; Roche, B.<sup>3</sup>; Kayser, Y.<sup>4</sup>; Guégan, J.F.<sup>3</sup>; Renaud, F.<sup>3</sup>; Thomas, F.<sup>3</sup>; Gauthier-Clerc, M.<sup>4</sup>; **van der WERF, S.<sup>5</sup>**

<sup>1</sup>GEMI, UMR CNRS/IRD 2724, Centre de Recherche de la Tour du Valat, France;

<sup>2</sup>GEMI, UMR CNRS/IRD 2724, Institut Pasteur URA3015 CNRS, France; <sup>3</sup>GEMI, UMR CNRS/IRD 2724, IRD, France; <sup>4</sup>Centre de Recherche de la Tour du Valat, France;

<sup>5</sup>Institut Pasteur, URA3015 CNRS, EA302 Université Paris-Diderot, France

The Camargue is an alluvial wetland covering some 140 000 ha in the Rhône delta (southern France). It is situated at the crossroads of numerous migratory routes of Palaearctic birds and is recognized as one of the main Mediterranean wintering areas. Wild birds, and especially waterbirds in the Anseriformes and Charadriiformes orders, are considered to be the natural reservoir for avian influenza viruses (AIV). We combined population surveillance, molecular analysis and modeling in order to answer simple questions such as: which are the bird species involved in the transmission of AIV? Did the highly pathogenic H5N1 virus circulate in the Camargue in winter 2005-2006, during its spread from Asia to Europe? How does AIV transmission and persistence among wild birds occur? Since fall 2005, we sampled more than 5000 birds belonging to 112 different species. Based on detection by real-time RT-PCR targeting the M gene, the level of infected birds was found to be about 3% overall and concerns only waterbirds (ducks and gulls). Highly pathogenic H5N1 viruses were not detected despite their occurrence during the same period in a nearby site in southern France. The circulating AIVs belonged to various subtypes including low pathogenic H5 viruses. First results concerning sequencing and phylogenetic analysis of circulating AIVs suggest possible cases of natural intercontinental exchanges and gene segment reassortment. Modeling of the dynamics of wild bird populations and AIV circulation point to the crucial role of the environment for transmission and persistence of AIV among wild birds. We present here the first data on the global pattern of avian influenza viruses' circulation among the wild bird communities present in the Camargue.

8-006

### Prevalence of influenza A virus in wild birds in Norway during Fall 2005-2007

**Germundsson, A.**; Madslien, K.; Hjortaas, M.J.; Handeland, K.; Jonassen, C.M.

National Veterinary Institute, Norway

Birds of wetlands and aquatic environments constitute the major natural influenza A virus reservoir. The birds may shed large amount of virus in their feces upon infection (Webster *et al.*, 1992). Influenza A virus of all hemagglutinin (HA) and neuraminidase (NA) subtypes (H1-H16 and N1-N9), have been identified in the wild bird reservoir, in particular birds belonging to Anseriformes (ducks, geese and swans) and Charadriiformes (gulls, terns and shorebirds). Many of the birds belonging to Anseriformes and Charadriiformes are known to perform short local movements and more extensive migrations, and thus potentially distribute influenza viruses between countries or even continents. Although influenza virus normally does not cause any disease in wild birds, they may cause serious disease outbreaks when introduced into poultry flocks. Prevalence of influenza A virus in their natural hosts depends on geographical location, seasonality and species (Krauss *et al.*, 2007, Munster *et al.*, 2007).

To determine the prevalence of different subtypes of influenza A viruses in Norway, virological surveillance was carried out in wild birds from five counties located in three different regions of the country: Hedmark and stfold (Eastern Norway), Rogaland (Western Norway) and S r- and Nord-Tr ndelag (Central Norway) from 2005 to 2007. These counties/regions are known to have both important resting sites for migrating birds and a high density of poultry. In total, samples from 3144 healthy birds (1940 ducks, 1204 gulls) shot during the ordinary hunting season (August-December) 2005-2007 were examined. Only cloacal swabs were collected from 1055 of the birds, whereas from the remaining birds the sampling included both tracheal and cloacal swabs. For detection of influenza A virus, real-time RT-PCR was performed using general influenza A virus primers and TaqMan probes, specific for the matrix gene sequence (Spackman *et al.*, 2002). The samples found to be positive in the initial pan-influenza A virus real-time RT-PCR, were further subtyped, using a specific H5 real-time RT-PCR (Spackman *et al.*, 2002), and RT-PCRs for the HA2 and full-length NA genes (Phipps *et al.*, 2004, Hoffmann *et al.*, 2001).

In total, 342 (10.9%) of the birds sampled were positive for influenza A virus. The prevalence in ducks was 13.9% (270/1940) and peaked in October each year. This peak in October is likely to be linked to a high concentration of birds at moulting and resting sites during this month where the infection can be easily transmitted to a high proportion of juvenile ducks. Juvenile birds are immunologically naïve and therefore probably more susceptible to influenza A viruses (Fouchier *et al.*, 2005). The

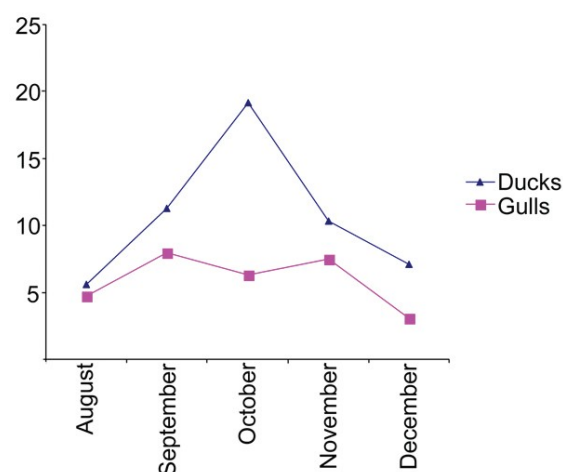
## POSTER PRESENTATIONS

prevalence of influenza A virus in gulls was 6.0% (72/1204) and no clear peak was observed during the sampling period. In addition to the 3144 ducks and gulls collected, 224 samples from geese and waders were collected. None of the geese or waders was infected with influenza A virus.

One Herring Gull, 19 Mallards, four Common Teal and one Wigeon were found to carry the low pathogenic (LPAI) H5 subtype. The HA subtype was determined in 158 of the 342 positive influenza samples. The HA subtypes identified in ducks were H1 (9.1%), H2 (3.5%), H3 (11.3%), H4 (7.8%), H6 (24.1%), H8 (0.14%), H9 (15.6%), H10 (1.4%), H11 (1.4%) and H12 (6.4%). HA subtypes isolated from gulls were H4 (11.8%), H6 (11.8%), H13 (35.3%) and H16 (41.2%). Our data indicate that influenza A virus subtypes H1-H12 are mainly found in ducks whereas subtypes H13-H16 occur in gulls as previously reported (Munster *et al.*, 2007).

The most commonly occurring HA subtype in 2005 in the present study (as earlier reported by Jonassen and Handeland, 2007), was H6 (36%, 39/80), while H4 (16.9% 10/59) dominated in 2006. Interestingly, subtype H4 was not at all detected in the 2005 screening. One plausible explanation for this difference between years could be a cyclic pattern of occurrence of different subtypes of the virus, as reported in other studies (Fouchier *et al.*, 2007). This study found a prevalence of influenza A virus in Norway that was higher than reported from North America, and in other European studies except for Sweden (Krauss *et al.*, 2007, Munster *et al.*, 2007). This higher prevalence in Scandinavian birds presumably is attributed to an ecological system with breeding areas and temperatures favouring virus replication and transmission.

Figure 1. Prevalence of Influenza A virus in ducks (triangles) and gulls (squares) in Norway during fall migration 2005-2007.



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8-007

### Prevalence of avian influenza viruses in waterfowl and terrestrial birds in Slovakia

Betakova, T.<sup>1</sup>; Gronesova, P.<sup>1</sup>; Mizakova, A.<sup>2</sup>; Kabat, P.<sup>3</sup>; Trnka, A.<sup>4</sup>

<sup>1</sup>Institute of Virology, Slovakia; <sup>2</sup>Military Hospital, Department of Hygiene, Epidemiology, Veterinary Provision And Laboratory Diagnostic, Liptovsky Mikulas, Slovakia; <sup>3</sup>Comenius University, Department of Microbiology and Virology, Bratislava, Slovakia; <sup>4</sup>Trnava University, Trnava, Slovakia

The prevalence of avian influenza virus (AIV) infections, together with the distribution of different AIV subtypes, was studied in migratory waterfowl and terrestrial birds caught in three localities in Slovakia during 2006 and 2007. Oropharyngeal and cloacal swabs were tested by RT-nested PCR. Samples obtained from waterfowl captured in the Senianske Ponds area of Eastern Slovakia showed the highest diversity of AIV isolates. A total of 13 different subtypes were detected in 19 samples from this location (H1N2, H2N2, H3N2, H6N6, H7N6, H9N2, H9N5, H9N6, H10N5, H10N6, H12N6, H13N6, and H16N6). H3N5 virus was

detected in 50% of passerines testing positive for AIV in the Parizske Wetlands, with H7N2, H9N2, H9N5, H12N1, and H13N2 infections also recorded at this locality. H9N5 virus predominated in passerines captured at Trnava Ponds, with isolates H1N6, H6N5, H7N2, H7N6, H10N3, H10N6 also detected at this location. There were five cases where different AIV infections were detected in oropharyngeal and cloacal samples originating from the same bird (H13N6 and H1N<sup>2</sup>; H10N5 and H12N<sup>6</sup>; H9N5 and H6N<sup>5</sup>; H10N6 and H7N<sup>6</sup>; H9N2 and H3N5 in the oropharynx and cloaca, respectively). This research was supported by the Slovak Research and Development Agency, grant No. APVV-51-004105 and by the VEGA- Grant Agency of Science, grant No.2/6152/06.

8-008

### Detection of avian influenza virus in wild pigeons

**Mizakova, Adriana<sup>1</sup>**; Gronesova, P<sup>2</sup>; Betakova, T.<sup>2</sup>

<sup>1</sup>Comenius University, Slovakia; <sup>2</sup>Institute of Virology, Slovakia

During spring 2006, we screened 25 pigeons for the presence of avian influenza virus (AIV). The pigeons were caged randomly in the city centre of Kosice, one of the metropolises of the Slovak Republic. The cloacal and throat swabs were analyzed by PCR. Our screening approach revealed that 24% of pigeons were positive for AIV. Regarding the AIV, three different subtypes were identified: H7N3, H9N5 and H14N8. This study demonstrates that wild pigeons represent a reservoir of important zoonotic agents. This research was supported by the Slovak Research and Development Agency, grant No. APVV-51-004105.

8-009

### Assessment of the inactivation of H5N1 avian influenza virus with different agents

**Mihai, Maria Elena**; Tecu, C.; Alexandrescu, V.I.; Sbarcea, C.E.; Baetel, A.E.; Necula, G.; Lupulescu, E.; Onu, A.

"Cantacuzino" National Institute for Research and Development in Microbiology and Immunology, Romania

H5N1 represents, today, a real threat to Romania. The country was confronted with two waves of avian flu with highly pathogenic avian influenza (HPAI): the first wave in October 2005 started from Ceamurlia-de-Jos, (Tulcea county- Danube Delta), in small backyard premises, while the second wave in May 2006 started

from Codlea (Brasov county) in a commercial farm of chickens. An outbreak in backyard poultry was recorded in November 2007 in Murighiol (Tulcea county- Danube Delta). The source was the residues of an infected coot hunted in the Danube Delta. The Danube Delta is an important station on the route of migratory wild birds, which could become an endemic area for the avian influenza viruses, with the potential for outbreaks in poultry and even transmission to humans. Since there is little information about the H5N1 avian influenza virus survival in the environment, the objective of our study is to find the best combinations of chemicals, in different pH conditions, salinity and temperature that impact upon the virus survival. Thus as the first step, we tested different commercial chemical agents in order to estimate the extent of H5N1 avian influenza virus inactivation. Study design: the tests were carried in BSL 2+ conditions using a stock of H5N1 – NIBRG – 14 (with haemagglutinin - HA and neuraminidase – NA genes derived from A/Viet Nam/1194/2004 – clade 1), prepared in SPF embryonated eggs. Virus concentrations used in the experiments were high, medium and low (calculated by EID<sub>50</sub>). The commercial disinfectants were selected according with the method of action, low cost and toxicity belonging to four groups: chlorine and chlorine compounds containing 2.5 g sodium dichloroisocyanurate (NaDCC); oxidizing agents: pentapotassium bis(peroxymonosulphate) bis(sulphate); aldehydes - 2.2% glutaraldehyde; alcohols: ethanol and different concentrations of isopropanol. The experiments were performed at different temperatures (4 oC; 22 oC; 37 oC) and different exposure times (recommended by the manufacturer), followed by neutralization of the disinfectant. As a substrate to monitor the retention infectivity of the influenza virus, we used two or three passages in embryonated chicken eggs (4 eggs/each sample). Virus detection in the allantoic fluids was made by haemagglutination and in addition we developed and tested a qRT-PCR assay. Every experiment was carried out with virus and disinfectant controls. Our preliminary results indicate that NIBRG – 14 is sensitive to the action of disinfectants used and further stages of the study will be carried out on samples collected from the environment (water, soil and bird feces ).

## POSTER PRESENTATIONS

8-010

### Detection of avian neutralizing antibodies using a pseudotype virus neutralization assay

**Sahlin, Sofie**<sup>1</sup>; Falk, K.I.<sup>1</sup>; Olsen, B.<sup>2</sup>; Temperton, N.J.<sup>3</sup>; Lundkvist, Å.<sup>1</sup>

<sup>1</sup>Karolinska Institutet, Department of Microbiology, Tumor and Cell Biology, Sweden; <sup>2</sup>Kalmar University, Section for Zoonotic Ecology and Epidemiology, Sweden; <sup>3</sup>MRC/UCL Centre for Medical Molecular Virology, Division of Infection and Immunity, UK

Serological and sequential analysis of highly pathogenic avian influenza (HPAI) H5 strains, isolated from humans and birds, reveal that the H5 strains responsible for outbreaks of HPAI that have occurred since 1997 in Hong Kong are genetically separated. The strains can be divided into several clades and also further divided into distinct sub-clades. Vaccine trials and other research indicate that there is little or no cross-neutralization between the different sub-clades of the HPAI subtype H5, meaning that HPAI viruses of subtype H5 evolve fast and differ significantly in antigenicity. In contrast, when studying low pathogenic avian influenza virus strains of subtype H5 circulating in wild birds, there are very small differences between them, indicating low evolutionary pressure on these forms of the virus. The low pathogenic avian influenza virus variants are adapted to their host and persistence within wild bird populations is not dependent on rapid evolutionary change. We now aim at clarifying whether or not avian antibodies targeting avian low pathogenic H5 are capable of neutralizing highly pathogenic forms of H5 influenza. Recently, a reliable and safe neutralization test for HPAI H5N1 was developed, making it possible to perform an H5N1 neutralization test at biosafety level 2. The assay relies on retroviral pseudotypes bearing only the influenza A H5 hemagglutinin on the surface of the pseudotype progeny virus, making this a safe, reliable, sensitive and specific assay for the detection of H5N1 neutralizing antibodies.

8-011

### Avian influenza viruses detected in nestlings of European Magpie (*Pica pica*)

**Mizakova, Adriana**<sup>1</sup>; Betakova, T.<sup>2</sup>; Gronesova, P.<sup>2</sup>; Trnka, A.<sup>3</sup>; Kabat, P.<sup>1</sup>

<sup>1</sup>Comenius University, Slovakia; <sup>2</sup>Institute of Virology, Slovakia; <sup>3</sup>Trnava University, Slovakia

European Magpie (*Pica pica*), a medium-size passerine bird from the crow family (Corvidae), is a common resident breeder evenly distributed in urban, rural and natural open landscapes and prefers to nest near towns, villages, farms, and recently in

strips of green along the highways and railways. While these birds are susceptible to infection with AIV, they can carry these viruses with no apparent signs of harm. We were studying the prevalence of AIV among European Magpie and especially the possible transmission of AIV to their nestlings. The oropharyngeal and cloacal samples were collected from 1-9 day old nestlings in the nests around Trnava, in April 2007. The nests were 500 m - 2 km from each other. The results showed that at least 50% of nestlings from each nest, except one nest number, were positive for AIV. In most cases, virus H7N2 was identified in all infected nestlings. However, there were nests where different viruses were found, H2N2 and H9N1, respectively. This research was supported by the Slovak Research and Development Agency, grant No. APVV-51-004105.

8-012

### Tenacity of avian influenza A-viruses

**Waehlich, S.**; Hudewenz, N.; Pauli, G.; Dupke, S.; **Schweiger, B.**

Robert Koch Institute, National Reference Centre for Influenza, Berlin, Germany

Although bird flu is a theme of big public interest, there is a lack of knowledge about the survival abilities of avian influenza viruses outside the host organism. However, with the threat of possible new infections, it is especially important to have data about the tenacity of avian influenza viruses under different environmental conditions. In this study, the survival abilities of low pathogenic avian influenza viruses (LPAIV) and highly pathogenic avian influenza viruses (HPAIV) were investigated. As LPAIV we selected two strains representing the subtypes H5N6 and H7N1 and two H5N1 viruses isolated in Germany in 2006 as representatives of HPAIV. We focussed our tests on environmental materials that play an important role in cases of bird flu outbreaks. Therefore, chicken meat, Baltic Sea water, chicken faeces and physiological buffer as a control were contaminated with the LPAIV and HPAIV strains. Since both different materials and the temperature have a great influence on the tenacity, the contaminated samples were stored at room temperature, at 4°C and at -20°C in constant humidity conditions. Our results show that particularly at room temperature there are differences in the tenacity between the four tested virus strains. Especially in Baltic Sea water and chicken meat, the highly pathogenic virus strains remain infectious much longer (about two to three times longer) than the low pathogenic virus strains. We also found differences in the survival abilities of the virus strains under the tested environmental conditions, up to 130 days. Particularly at low temperatures, all tested virus strains showed longer infectivity than at room temperature. Because of the long survival times most of the tests under cold conditions are still not completed after test duration up to 320 days. In

conclusion, we could show that the survival periods differ from strain to strain with an unexpected high ability to survive at low temperatures (4°C, -20°C). Our results contribute to more comprehensive knowledge on the tenacity of avian influenza viruses and thus allow for better judgement on necessary control measures.

8-013

### Sequence characterization of a porcine avian-lineage H1N1 influenza virus isolated from a human sample

**Schweiger, Brunhilde<sup>1</sup>; Heckler, R.<sup>2</sup>; Biere, B.<sup>1</sup>**

<sup>1</sup>Robert Koch Institute, Germany; <sup>2</sup>Public Health Department of Lower Saxony, Germany

In March 2007, an influenza virus was isolated from a male 17-year-old patient in Lower Saxony showing symptoms of influenza-like illness. This isolate (A/Niedersachsen/58/07) was positive in a generic influenza A real-time PCR and gave high titers in a hemagglutination test. However, real-time PCR assays for human hemagglutinin subtypes H1 and H3 or human neuraminidase subtypes N1 and N2 remained negative, and also no reaction was observed in a hemagglutination inhibition test with ferret sera specific for current human reference strains. Sequencing of a short M gene fragment suggested a porcine origin of the virus, which could be affirmed by sequencing of all eight genome segments with degenerate PCR primers. The isolate resembles the avian lineage of porcine H1N1 viruses, which was introduced into the pig population in the 1970s and completely replaced the classical H1N1 lineage in Europe. All eight genome segments showed the highest homology to the avian lineage viruses, thus no reassortment with human viruses and other porcine subtypes or lineages is apparent. The infection of humans with influenza viruses of porcine origin is a very rare event, which has been described only a few times in history. However, the simultaneous infection of an individual with a porcine and a human virus could result in new virus variants with HA and NA subtypes to which the human population is serologically naïve, thus possibly sparking a pandemic. As a consequence, all human infections with porcine viruses need to be carefully observed.

8-014

### Highly pathogenic avian influenza virus H7N7 isolated from a fatal human case causes respiratory disease in cats, but does not spread systemically

**van Riel, Debby<sup>1</sup>; Rimmelzwaan, G.F.<sup>2</sup>; van Amerongen, G.<sup>2</sup>; Fouchier, R.A.M.<sup>2</sup>; Osterhaus, A.D.M.<sup>2</sup>; Kuiken, T.<sup>2</sup>**

<sup>1</sup>Erasmus MC - Department of Virology, Netherlands; <sup>2</sup>Erasmus MC, Netherlands

Previously we have shown that highly pathogenic avian influenza virus (HPAIV) of the subtype H5N1 is able to cause severe disease in cats. Virus replication was not only restricted to the respiratory tract but also occurred in tissues belonging to the nervous, cardiovascular, urinary, digestive lymphoid and endocrine systems. In order to investigate if infection and systemic spread in cats is a common feature of all HPAIV or a specific feature of the HPAIV H5N1, we infected domestic cats with a HPAIV H7N7. This virus was isolated from a fatal human case during the 2003 outbreak in the Netherlands. Three domestic cats were infected intratracheally and euthanized 7 days post-infection. Oral and rectal swabs were taken daily; during necropsy, samples were taken for virus isolation and histology. All cats became infected with H7N7 virus. Virus replication (detected by virus isolation and immunohistochemistry) was restricted to the respiratory tract. To determine whether these differences between H5N1 and H7N7 viruses were due to differences in pattern of viral attachment, we performed virus histochemistry (with H5N1 and H7N7 viruses) on tissues of uninfected cats. In the tissues of the respiratory tract, liver and central nervous system, there were no major differences observed in sites and cell types to which H5N1 and H7N7 viruses attached. These data show that HPAIV H7N7 was able to infect and cause severe respiratory disease in cats but – in contrast to H5N1 virus – did not spread systemically. Both viruses are highly pathogenic for poultry and can cause fatal respiratory disease in humans. Further investigations are required to determine which factors contribute to the differences in tissue tropism of these two virus infections in cats.

8-015

# **Genetic and antigenic characterization of swine influenza virus in France: Identification of novel H1N1 reassortants**

**Kuntz-Simon, G.<sup>1</sup>**; Franck, N.<sup>1</sup>; Quéguiner, S.<sup>1</sup>; Gorin, S.<sup>1</sup>; Eveno, E.<sup>2</sup>; Madec, F.<sup>2</sup>

<sup>1</sup>AFSSA-LERAPP, Swine Virology Immunology Unit, France; <sup>2</sup>AFSSA-LERAPP, Pig Epidemiology and Welfare Unit, France

Swine influenza is a highly contagious viral disease of the respiratory tract in pigs. Besides their veterinary health interest, swine influenza virus (SIV) infections are also a matter of deep concern due to the possible pathogenic transmission to humans. Pigs are susceptible to infection with both avian and human influenza viruses. They could serve as an intermediate host for the adaptation of avian influenza viruses to the mammalian host, as well as for the generation of pandemic viruses through reassortment. Three major subtypes of influenza virus A, H1N1, H3N2 and H1N2, co-evolve in pigs worldwide. However, various lineages of each subtype can be distinguished depending on the world area. In Europe, H1N1 SIV originated from the transmission of an avian influenza virus to pigs in 1979. H3N2 strains have circulated in European pigs since the mid 1980s and are reassortants between an H3N2 strain of human origin and a swine avian-like H1N1 strain from which they inherited the internal genes. In the early 1990s, H1N2 viruses arose by genetic reassortment of human H1N1 viruses and swine reassortant H3N2 strains. Thus, H1N2 viruses possess a haemagglutinin (HA) gene closely related to that of human H1N1 strains that were circulating in the late 1970s. No report has been done to characterize circulating SIV strains in France since 2000. In order to guarantee an effective epidemiological surveillance in this country, we examined genetic and antigenic variation in SIV isolated from 2000 to 2007 in pigs in Brittany, the leading pig-producing region. SIV of H1N1 and H1N2 subtypes are currently circulating in Brittany in equal proportions, but no H3N2 strain could be isolated. Genetic comparisons of HA1 genes showed a marked heterogeneity among H1N2 strains possessing human-like HAH1, which contrasted with the high similarity observed among avian-like H1N1 viruses. Genome sequencing revealed for four strains the novel combination of the human-like HAH1 gene of H1N2 viruses and the NAN1 gene of avian-like H1N1 viruses. Sequencing of the six internal genes showed that they were all of avian origin, closely related to those of avian-like H1N1. Three H1N1 reassortants were isolated in 2001 and 2005 in the same farm and were genetically and antigenically closely related. By contrast, the fourth one, isolated in 2006 in another farm, presented a particular antigenic reaction pattern. Analysis of its HA deduced amino acid sequence revealed it had a deletion of one residue belonging to the receptor-binding pocket, a deletion that was also observed in recent human H1N1 strains. Identification

of these novel H1N1 reassortants highlights the importance of continuous disease-based surveillance in order to monitor their evolution, their possible adaptation to the pig population and their increased chances of transmission to humans.

8-016

# **Comparison of the transmission dynamics of high pathogenicity (H7N1) avian influenza A virus in poultry species**

**Essen, Stephen<sup>1</sup>**; Gardner, R.<sup>1</sup>; Outtrim, L.<sup>1</sup>; Brookes, S.M.<sup>1</sup>; McCauley, J.W.<sup>2</sup>; Iqbal, M.<sup>3</sup>; Brown, I.H.<sup>1</sup>

<sup>1</sup>Veterinary Laboratories Agency (VLA), UK; <sup>2</sup>National Institute for Medical Research, UK; <sup>3</sup>Institute for Animal Health, UK

Avian influenza poses a serious threat to animal and human populations and provides an unusual opportunity to synthesize ecological and evolutionary pathogenic dynamics. A key objective is to understand the transition from influenza in aquatic birds, where virus is highly diverse and generally has a low pathogenicity (LPAI) to frequently much more dangerous infections in poultry. Of paramount importance to poultry is infection by highly pathogenic avian influenza (HPAI) viruses, which can result in mortality approaching 100%. The transmission dynamics of H7N1 HPAI isolates from an outbreak in N. Italy in 1999-2000 in chickens, turkeys and ducks was studied. Using a 10 infected (buccal cavity) plus 10 naïve contact bird model and a dose of 10<sup>6</sup> EID<sub>50</sub>/0.1ml, mortality in turkeys was 100% both in infected birds (40-64 hours) and naïve contacts (64-96 hours), in chickens this was 95% in infected birds (48-96 hours), but there was no transmission, and in ducks no mortality was observed in either infected or contact birds even though infection (100%) and transmission (70%) was established. The dissemination of disease, as indicated by the number of birds and level of virus being excreted (buccal and cloacal) was similar in pattern, however, disease was greatest in turkeys, then chickens, then ducks. In infected turkeys, 100% of the birds were shedding virus in the buccal cavity by 16 hours post-infection and the rate of shedding peaked between 24-40 hrs. All birds were excreting virus from the cloaca by 32 hours and it peaked between 40-48 hours. All in-contact birds were infected by 40 hours and shedding peaked at 64 hours. In infected chickens, 80% of birds were shedding from the buccal cavity by 24 hours and 100% by day two. Excretion from the cloaca occurs in 35% at day one and 95% by day two. The level of virus shed from the buccal cavity is constant over days one and two, whilst cloacal shedding peaked at 48 hours. In infected ducks, 100% of the birds shed from the buccal cavity between days 1 and 11, 50% shed from the cloacal

cavity between days 2-9, both peaking at day 5. In the contact ducks, 30% shed from the buccal cavity from days 9-11 and 40% excreted from the cloaca from days 6-10 (60% one or the other, or both). Peak shedding was recorded at day 9 for both routes. The transmission dynamics of H7N1 HPAI isolates in chickens, turkeys and ducks were substantially different. Turkeys were the most susceptible followed by chickens and ducks. Shedding was greatest in the most susceptible species but of shorter duration as a result of morbidity, duration and mortality rates. In turkeys and chickens, shedding was predominantly buccal, where as in ducks it was mainly via the cloacal route. The differences in the route, level and duration of viral shedding between the infected species may allow selective replication advantages for virus adaptation within host and onward transmission, particularly in ducks.

8-017

#### Lack of transmission of low pathogenic avian influenza viruses between pigs and from pigs to ferrets

**De Vleeschauwer, A.;** Braeckmans, D.; Van Poucke, S.; Barbé, F.; Van Reeth, K.

Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium

**Introduction:** Pigs have been shown to be susceptible to both human and avian influenza (AI) viruses (1,2). Moreover, pigs are considered as a potential intermediate host for the transmission of avian influenza viruses from birds to humans. However, highly pathogenic (HP) H5 and H7 AI viruses failed to transmit between pigs in experimental studies (3-6). Transmission of AI viruses from pigs to humans has never been documented. The aim of this study was to examine the capacity of different low pathogenic (LP) AI viruses to be transmitted between pigs and from pigs to ferrets. Ferrets are susceptible to a wide range of influenza viruses and are frequently used as a model for humans in influenza research.

**Materials and methods:** Seventy-two six-week-old pigs and twelve adult ferrets were used. All animals were influenza seronegative. An H1N1 and an H3N2 swine influenza virus (SIV) and four LPAI viruses of various hemagglutinin subtypes (see table 1) were used in six separate experiments. The LPAI viruses were previously shown to replicate in experimentally inoculated pigs and ferrets. In each experiment, six pigs were inoculated intranasally with  $7.0 \log_{10}$  EID<sub>50</sub> of the respective virus. Two days later, six contact pigs were housed in direct contact with the inoculated pigs. At the same time two ferrets were placed in a wire cage in each pig stable, allowing aerogenic contact. All animals were monitored daily for clinical signs. Nasal swabs for virus titration were collected from 0 until ten days post-inoculation

(DPI) or post-contact (DPC). Serum was collected from all animals at 0, 14 and 28 DPI or DPC and examined for antibodies against the homologous virus in an immunoperoxidase monolayer assay (IPMA). During the whole experiment, the stable environment was maintained at a temperature of 20-22 °C and a relative humidity of 50-70%, mimicking field conditions.

**Results:** An overview of the results is given in table 1. None of the H1N1 or H3N2 SIV inoculated pigs showed clinical signs. All inoculated and contact pigs excreted virus and seroconverted (IPMA antibody titre ranging from 256 to  $\geq 8192$ ). All SIV contact ferrets shed virus for up to six consecutive DPC and had antibody titres  $\geq 8192$  at 14 DPC.

After inoculation of pigs with the LPAI viruses, no clinical signs were observed. All H3N6 and H4N1 inoculated pigs, one out of six H5N1, and five out of six H7N1 inoculated pigs shed virus for a variable duration (1 up to 6 consecutive DPI). The remaining pigs did not shed virus. All inoculated pigs showed seroconversion against the homologous virus at 14 DPI (IPMA antibody titres ranging from 2048- $\geq 4096$ , 256-2048, 64-512 and 128- $\geq 4096$  for H3N6, H4N1, H5N1 and H7N1 inoculated pigs respectively). No virus excretion was detected in nasal swabs of any of the contact pigs. No serological response was detected in the H3N6, H4N1 and H5N1 contact pigs. Only two out of five H7N1 contact pigs seroconverted (IPMA antibody titres 64 and 512 at 14 DPC). None of the LPAI contact ferrets shed virus or showed a serological response.

**Discussion:** Our data indicate that the examined LPAI viruses transmit inefficiently between pigs and generally fail to transmit from pigs to ferrets. From the four LPAI viruses tested in this study, only one spread to a very limited degree between pigs and none were transmitted to ferrets. These findings are in agreement with reports of the failure of HPAI viruses to spread between pigs or ferrets (3-7), and suggest a minimal risk of transmission of LPAI viruses via pigs to humans.

The reason for the lack of transmission of LPAI viruses between pigs and from pigs to ferrets remains obscure. Virus titres in nasal swabs of LPAI inoculated pigs were generally lower and more variable than those of the SIV inoculated pigs. The lower virus dose to which the contact animals were exposed may be an important factor for the lack of transmission, since it is known that high inoculation doses are needed to experimentally infect pigs with AI viruses (2). Serological evidence of virus spread to contact pigs was found only with the H7N1 isolate, but the H7N1 contact pigs did not excrete virus. However, virus excretion titres of the H7N1 inoculated pigs were similar to those of pigs inoculated with the other LPAI viruses. Our results are in line with the assumption that AI viruses have to undergo genetic changes to adapt to a mammalian host and to spread efficiently between mammals. It is noteworthy in this regard that the H7N1 virus was isolated from chickens, while the three other LPAI viruses examined were isolated from wild ducks. It is possible therefore that the chicken virus is more adapted to replication in mammals than the viruses from wild ducks. However, further research is needed to examine this possibility. We now have a suitable model to examine the

## POSTER PRESENTATIONS

transmission capacity of AI viruses with well defined genetic differences between pigs and from pigs to ferrets.

Table 1. Transmission of swine- and avian influenza virus isolates from inoculated pigs to contact pigs and contact ferrets

Virus used for inoculation	Number of animals with					
	Inoculated pigs		Contact pigs		Contact ferrets	
	Virus excretion	Serol. response	Virus excretion	Serol. response	Virus excretion	Serol. response
A/swine/Belgium/1/98 H1N1	6/6	6/6	6/6	6/6	2/2	2/2
A/swine/Flanders/1/98 H3N2	6/6	6/6	5/5 <sup>1</sup>	5/5 <sup>1</sup>	2/2	2/2
A/duck/Belgium/06936/05 H3N6	6/6	6/6	0/6	0/6	0/2	0/2
A/mallard/Alberts/47/98 H4N1	6/6	5/5 <sup>2</sup>	0/6	0/5 <sup>2</sup>	0/2	0/2
A/mallard/Italy/3401/05 H5N1	1/6	6/6	0/6	0/6	0/2	0/2
A/chicken/Italy/1067/V99 H7N1	5/6	6/6	0/6	2/5 <sup>2</sup>	0/2	0/2

<sup>1</sup>: only five pigs available; <sup>2</sup>: one pig died before 14 DPI or DPC

### Acknowledgements:

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8-018

## Influenza A viruses binding pattern in wild birds: towards a better understanding of virus circulation in natural reservoirs

Jourdain, E.<sup>1</sup>; van Riel, D.<sup>2</sup>; Munster, V.J.<sup>2</sup>; Kuiken, T.<sup>2</sup>; Olsen, B.<sup>1</sup>; Osterhaus, A.D.M.<sup>2</sup>; Ellström, P.<sup>1</sup>

<sup>1</sup>Kalmar University, Sweden; <sup>2</sup>Erasmus Medical Center, Netherlands

Influenza A viruses (IAVs) have a broad spectrum of natural hosts among mammals and birds. Molecular interactions between the different viral hemagglutinins and the sialic acids displayed at the surface of host cells are thought to play a key role in the specificity of virus – host interactions because attachment to host cells is the first step necessary for multiplication of the virus. The distribution of target molecules for influenza viruses have been described for several mammal species (including human, horse, pig, dog, mouse, ferret and cat). Conversely, although birds are the main reservoir of IAVs in nature, knowledge about the expression pattern of target molecules in bird tissues is currently limited to very few species, namely chicken, mallard and quail. Because avian species are highly diverse genetically and in their ecology, differential co-evolution history and therefore great variations in the expression of IAV receptor molecules might be expected. In this study, we investigate the expression of target molecules for IAVs in the tissues of various bird species using both lectin staining and virus histochemistry methods. The results are analyzed along with observed prevalence data and provide new insights into the circulation of IAVs in wild birds. In the current context of emerging strains pathogenic for humans, such a map of the receptors expressed by wild birds will prove highly valuable for better predictions of the potential spread of IAVs.

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## 9 MATHEMATICAL MODELLING

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9-001

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### The seroprotection threshold titre of 40 in the analysis of hemagglutination inhibition (HI) studies with influenza vaccines

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**Beyer, Walter;** Osterhaus, A.D.M.

*Erasmus MC Rotterdam, Netherlands*

As hemagglutination inhibition (HI) antibody is inversely related to the probability of influenza virus infection, HI antibody response following influenza vaccination serves as a serological surrogate marker for vaccine efficacy. In addition, a dichotomous surrogate of protection is often used: seroprotection. A subject is said to be seroprotected if the antibody response exceeds a certain threshold titre value, mostly 40. The protection threshold serves to calculate the (sero)protection rate, a statistic used in the analysis of studies with annual influenza vaccines, and currently also with experimental pandemic vaccines. In 1972, Hobson *et al.* defined the threshold as 'the titre at which the infection rate is reduced to half the maximum observed rate' (J.Hyg. 1972;70:767-777). This definition was later modified to 'a serum HI titre of 40 as the 50% protective level of antibody'. The silent assumption of this concept is that the protection threshold sharply discriminates between subjects with a (very) high probability of infection when exposed (pre-exposition HI antibody titre < 40) and subjects with a (very) low probability of infection ( $\geq 40$ ). We want to show that the threshold concept is meaningful only in the case of a mathematically ideal sigmoid curve with HI antibody as an independent variable and probability of infection as dependent variable. A pre-requisite of the ideal curve is the complete absence of protective factors other than HI antibody (innate, cellular, and other humoral immunity). However, when not an ideal design but rather clinical evidence is looked at (data from 19 challenge trials with 3,590 persons), it turns out that, on average, 25% of the population shows resistance against influenza infection even without HI antibody. This circumstance flattens the pertinent curve and diminishes the discriminating ability of the protection threshold. Therefore, the seroprotection rate can only poorly predict real protection.

9-002

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### Is neuraminidase really beneficial in influenza vaccines? A review of the literature

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**Beyer, Walter;** Osterhaus, A.D.M.

*Erasmus MC Rotterdam, Netherlands*

High serum anti-hemagglutinin antibody titres prevent influenza infection and illness, irrespective of the presence of other immune factors such as anti-neuraminidase antibody (aNAab). The vaccine dosage is exclusively determined by its hemagglutinin (HA) contents. The well-known drawback of this approach is the insufficient cross-protection of anti-HA antibody against drifted virus variants (antigenic mismatch) and vaccine failure. Earlier reports suggest a beneficial role of serum aNAab in the prevention of influenza illness. We have searched for data from published literature on the association between aNAab and influenza infection / clinical illness in humans, and identified ten studies, mainly from the 1970s and involving 1,731 persons (healthy children and young adults). All studies assessed infection, and all but one also clinical illness, after experimental or natural challenge with A-H3N2 strains. Eight out of 10 studies provided some evidence that anti-NA antibodies may prevent infection. Nine out of 9 studies convincingly showed that aNAab was associated with a decreased chance of clinical illness. These results are contrasted by the apparent failure of conventional vaccines during epidemics with mismatch. As NA exhibits a slower antigenic drift than HA, aNAab should provide second-line protection against drifted virus strains when anti-HA antibody fails. Brett & Johansson suggest that vaccines produced from entire influenza virions, simply contain an NA amount too little to induce sufficient aNAab (Virology 2005;339:273-280). Indeed, one single virion contains ~400 HA trimers, but only 50 NA tetramers; the number of HA molecules is thus ~6 times larger than that of NA molecules. In a vaccine dose of 15 µg HA, only traces of NA can be expected. Conclusion: The effects of aNAab should be studied in various age groups, in particular the elderly, and for influenza (sub)types other than A-H3N2. If this data confirms the beneficial evidence of aNAab established in young persons, then new strategies to improve the current influenza vaccines can be considered.

9-003

### Predicting the efficacy of influenza vaccines using haemagglutination-inhibiting antibody titres: Application to a novel influenza vaccine given by intradermal microinjection

Andre, P.; Coudeville, L.; Bailleux, F.; Weber, F.

Sanofi Pasteur, Lyon France, France

**Background:** Anti-haemagglutinin antibodies, measured by the haemagglutination inhibition (HI) assay, are essential in the host's immunological response to influenza infection. We present an application of a model providing estimates of protection against influenza according to HI titre for comparing the efficacy of two vaccines.

**Methods and Findings:** Using the published data from 15 studies in which 5899 subjects were analyzed and 1304 influenza cases reported, we developed a model that provides an estimate of the level of clinical protection against influenza for any HI titre level. The model showed that there was a strong and positive relationship between HI titre and protection, confirming that HI is a good surrogate marker of protection for influenza. We then used this model with individual HI titres from two randomized clinical trials in elderly adults (60+ years), that compared the immunogenicity of an inactivated influenza vaccine given by intradermal microinjection (ID) with that of a conventional influenza vaccine given intramuscularly (IM), to predict the gain in vaccine efficacy that could be obtained with ID versus IM. Overall, 2947 elderly subjects were vaccinated ID and 1349 IM. Higher individual titres with the ID vaccine observed in these two clinical trials resulted in a predicted increase in vaccine efficacy of 16.5% [95% CI: 12.9-20.8] from 49.0% [62.8-65.7] for the IM vaccine to 57.2% [54.3-59.8] for the ID vaccine.

**Conclusion:** Our modelling approach provides useful information for translating observed differences in immunogenicity into predicted relative differences in vaccine efficacy.

9-004

### Behavioural changes in response to pandemics in an evolutionary game setting

Poletti, P.<sup>1</sup>; Caprile, B.<sup>1</sup>; Ajelli, M.<sup>1</sup>; Pugliese, A.<sup>2</sup>; Merler, S.<sup>1</sup>

<sup>1</sup>Fondazione Bruno Kessler, Italy; <sup>2</sup>Department of Mathematics, University of Trento, Italy

Time evolution of epidemics and pandemics derive from the complex interplay between the dynamics of infection and the behaviour of affected individuals. As it is widely accepted, in fact, people change their behaviour and contact patterns in response to epidemics – in particular to highly lethal ones (Ferguson, 2007). Experience from the 1918 epidemics also indicates that characterizing such changes proves crucial for improving model realism (Bootsma and Ferguson, 2007). By better understanding behavioural changes during pandemics we may hope to enhance the effectiveness of containment/mitigation policies. Human behaviour is driven by evaluation of prospective outcomes deriving from alternative decisions and cost-benefit considerations (von Neumann and Morgenstern, 1947; Hofbauer and Sigmund, 1998). Past experience, response to the action of other individuals and changes in external conditions are the relevant factors in the balance, to which Game Theory provides a natural modelling framework. As a recent application, tools from both classical and evolutionary Game Theory have been employed to explain the variation over time of voluntary vaccination uptake (Bauch *et al.*, 2004; Bauch, 2005). In this work, we strengthen the adoption of Evolutionary Game Theory as a framework for the study of the infection-behaviour interplay. In particular, we exhibit a new model for the evolution of contact rates in a simple, compartmental SIR model. Connections with the classical Game Theory setting are drawn, showing an asymptotic reduction of our model, as the time scale of behavioural evolution goes to zero. We consider a population of individuals able to reduce the number of contacts spontaneously, as a defensive response to an epidemic (e.g., autoquarantine). Those individuals that reduce the contacts run a smaller risk of being infected (possibly, no transmission), yet pay a cost because of their increased isolation; individuals continuing normal life pay instead the whole contextual risk of infection, which is zero in absence of infection. Specifically, the behaviour of susceptible subjects is modelled as driven by a two-strategy Imitation Game Dynamics, with payoffs depending on the infection status, via the perceived risk of infection. The model is that of Fig. 1, where  $S(t)$ ,  $I(t)$ ,  $R(t)$  denote frequencies of susceptible, infected and removed individuals at time  $t$ .  $1-x(t)$  is the frequency of individuals playing the defensive strategy  $c$  at time  $t$ .  $\beta_d$  and  $\beta_c$  are the transmission rates of individuals playing mutually exclusive strategies  $d$  and  $c$ , respectively, and  $1/\gamma$  is the mean infection duration. Parameters  $m$  and  $\theta$  account for the risk of infection, the cost of reducing contacts and the relative time scales of behaviour and infection dynamics. The variation of

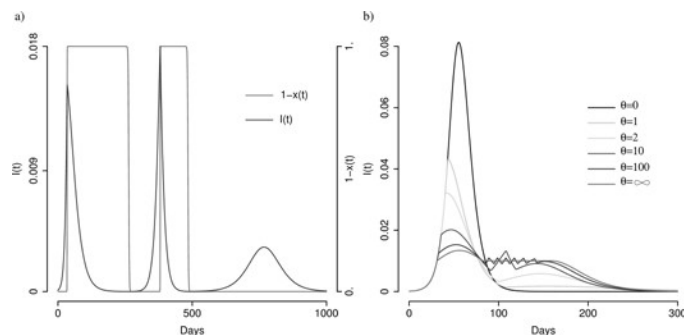
risk of infection during the epidemics may determine behavioural changes resulting in multiple waves (see Fig. 2a). Depending on the value of  $\theta$ , the model can also accommodate for different slopes along the increasing and the decaying phase of the same infection wave (Fig. 2b). Moreover, for suitable values of  $m$ , a family of trajectories is generated and converges to the one predicted by classical game theory, as the value of  $\theta$  grows to infinity (Fig. 2b). Finally, the model can be extended to account for more complex behavioural responses and different infection dynamics (for example, SIS models or seasonal epidemics). A further element of flexibility lies in the possibility of choosing among a variety of selection dynamics (e.g., Replicator-mutator; Adaptive dynamics (Nowak and Sigmund, 2004)). Let us stress here what is perhaps the most interesting feature of the approach we are proposing. Typically, behaviour and contact patterns are considered as “background” for the infection dynamics – i.e., they are not themselves variables of the dynamics. With the introduction of an explicit model for behavioural changes, the infection provides the context for the behavior and vice versa. In this way, (formal) symmetry between the two key factors is restored, and no by-principle prevalence is given to one factor over the other. This implies that not only do the dynamics of infection depend on both the transmission and behavior, but also the behaviour dynamics depend on behaviour (and infection as well). In fact, this is precisely what marks the difference between Evolutionary Game Theory and classical Game Theory, since the latter would result in (rational) instantaneous best responses to the infection dynamics, regardless of the current distribution of behavioural strategies. The class of models introduced in this paper may contribute to elucidate phenomena for which a behavioural basis is apparent, as in reaction to alerts (Wallinga and Teunis, 2004), or hypothesized, as for superspreading events (Lloyd-Smith, 2005). Also, empirical estimations of epidemic parameters (as, for example, the basic reproduction number) or the comparison between intervention strategies have to be carefully reconsidered whenever underlying behavioural dynamics are suspected.

Figure 1: The equation system

$$\begin{cases} \dot{S}(t) &= -[\beta_d S(t)x + \beta_c S(t)(1-x)] I(t) \\ \dot{I}(t) &= [\beta_d S(t)x + \beta_c S(t)(1-x)] I(t) - \gamma I(t) \\ \dot{R}(t) &= \gamma I(t) \\ \dot{x}(t) &= \theta x(t) [1-x(t)] [1-mI(t)] \end{cases}$$

Figure 2:

- The model accounts for multiple waves. Evolution of the fractions of infected individuals and individuals playing the defensive strategy  $c$  is shown over time.
- The model exhibits different slopes in the decay phase. Moreover, as  $\theta$  grows to infinity, the dynamics of  $I(t)$  converges to that prescribed by classical game theory.



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9-005

## Evaluating the effectiveness of antiviral treatment in models for influenza pandemic

Pugliese, Andrea; Lunelli, A.

University of Trento, Italy

The public health threat posed by novel strains of influenza gaining transmissibility in people and causing a human pandemic has stimulated a great development of mathematical models aiming at evaluating the effectiveness of potential control measures in containing the pandemic, and mitigating its effects.

Since it is unlikely that vaccines effective against the pandemic strains will be available until late in the pandemic spread, most mathematical models have studied the prophylactic use of antiviral drugs. Results of different studies are often in disagreement: while some authors draw positive conclusions about the possibility of slowing the spread of the infection and reducing the attack rate [3,4,8,10,12,13], even in circumstances in which a resistant strain spreads widely [11], others are more reluctant and suggest that a containment policy based on antivirals alone is unlikely to be successful [6,7,8].

Models used in previous studies range from simple deterministic compartmental models [2,9], to deterministic [8] or stochastic [4,5] metapopulation models, to microsimulations [6,7] that model individual behaviour and infection transmitting contacts. Despite this enormous difference in complexity, all the models share the same structure, with individuals progressing from Susceptibles to Exposed to Infectives to Removed, and antiviral therapies being administered. We believe that many differences found in previous analyses actually depend on details in modelling antiviral treatment, and that this can be best understood by studying simple SEIR models with antiviral treatment.

**Models and results:** We consider here deterministic SEIR-type models, all including the same antiviral strategy, but modelled in different forms, and compute the reduction of  $R_0$ , the infection reproduction ratio, obtained with treatment. All models assume that a fraction  $P$  (70% in the numerical examples) of the infected will be treated, that they will have their infectivity reduced to a factor  $r$  (20% in the numerical examples) and that their period of infectivity will be reduced by 1 day on average. Such figures are in the literature, but are only an example, since our results are concerned with the relative effect of different model assumptions; changing these numbers will change proportionately the effect in all models.

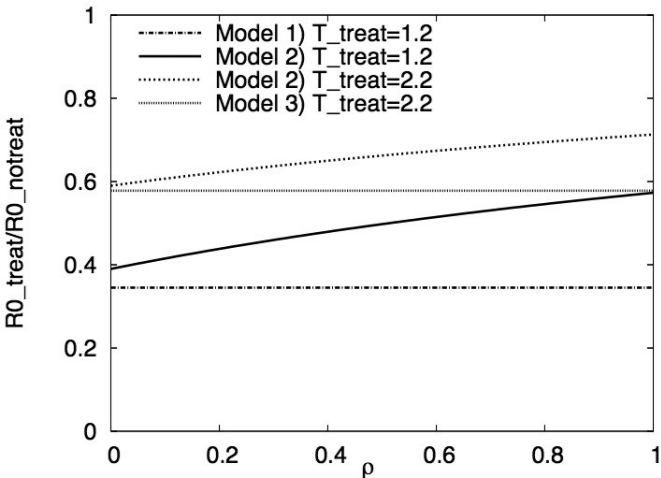
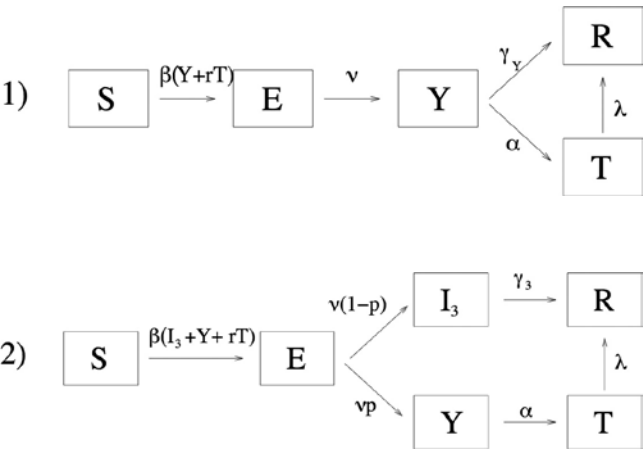
Antiviral treatment has been modelled in different ways. Some models assume that individuals receiving treatment may be distinguished from those that will not be treated (because, for example, of the absence of symptoms) and include therefore a class for infectives that will not be treated and one for those that eventually will. Other models assume that treated individuals are randomly picked among the infectives. In the simplest

framework, the two corresponding models are shown in the following figure: In both models,  $T$  represents the class of treated individuals, whose infectivity is reduced by a factor  $r$  while in Model 2,  $Y$  represents the infectives that do not receive treatment. We assume that their infectivity may be reduced (by a factor  $\rho$  varying between 0 and 1) as usual in case of asymptomatic infections (presumably less infectious). In the following Figure, we show the reduction of  $R_0$  entailed by the two models as a function of  $\rho$ , the factor of reduced infectiousness of class  $Y$ . In both models, 70% of the infectives are treated after on average 1.2 days, and the infectiousness of treated individuals drops to 20% of the original. It can be seen that, as expected, treatment is more effective ( $R_0$  is lower) the smaller  $\rho$  is (the infectiousness of individuals not reached by treatment); more surprisingly, the reduction is much higher for Model 1 (independently of parameter  $\rho$  which is not included in Model 1) than for Model 2: if untreated individuals are as infectious as treated ones ( $\rho=1$ ),  $R_0$  is reduced to 34% for Model 1 vs. 55% for Model 2; even if  $\rho=0$  (in Model 2 all infectious individuals get treated) Model 1 predicts a higher success of the therapy (reduction of  $R_0$  to 34% instead of 38%). This result comes from the fact that in Model 1 we are implicitly assuming that untreated individuals have a shorter infectious period than treated individuals. Since individuals in the class are subjected to two competing "risks" (recovery and treatment), individuals that escape treatment are on average those that have a shorter infectious period. This is a common feature of all models parameterised as Model 1.

The assumption of an average delay of 1.2 days between the start of the infectivity and the start of therapy is rather optimistic, considering that that infectiousness is assumed to be reduced as soon as therapy is started. The other two curves in Fig. 1 assume that therapy starts on average 2.2 days after the start of infectivity, while other parameters are kept the same; since in Model 1 this is not possible (there are only two parameters), we use instead Model 3 where the class  $I$  is split in two subsequent stages. In Model 3, treatment competes with natural recovery, like in Model 1; thus, it predicts a stronger effect of treatment than Model 2 with corresponding values.

**Discussion:** We have shown above that the effectiveness of antiviral treatment in containing a pandemic depends not only on the fraction of infectives that undergo therapy, by the effectiveness of the therapy in reducing the individual infectivity, but also very strongly on the timing of the therapy and the properties of the infectives that are not reached by treatment: Are they asymptomatic infectives with a lower infectiousness? Are they infectives that recover before treatment can reach them, as implicitly assumed in a class of models? Are they individuals as infectious as the other but unlikely (for social, behavioural or other reasons) to refer to physicians? The structure of a model includes assumptions on this, which can have a dramatic effect on the predicted effect of treatment: in Fig. 1, the most optimistic scenario predicts a reduction of  $R_0$  to 34%, the most pessimistic to 71%; if without treatment  $R_0$  were equal to 1.8, treatment may reduce  $R_0$  between 0.6 (very effective control) and 1.3

(some mitigation). These numbers are used only for illustration, since they are based only on very simplistic models and guess-estimates. Our point is mainly qualitative: beyond the need for realistic models of contact networks, an accurate modelling of how antiviral treatment is administered is also needed. The models shown here can be extended to cover variable infectivity [1], by using several infectious stages, as well as prophylaxis of household contacts, by modelling infection progression within a household, though the formulae will become more complicated. These computations of  $R_0$  are at the basis of infection spread also in more complex models. In metapopulation models, it is possible to compute a global reproduction ratio  $R_0$ , which determines the global course of infection, by a suitable average of the local  $R_0$ . In microsimulation models, because of individual variability and the clustering of infections, the expected numbers of cases potentially generated by an average individual cannot define  $R_0$  exactly but still drives the exponential initial growth of epidemics.



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9-006

Optimizing antiviral treatment and prophylaxis during an influenza pandemic

Merler, S.<sup>1</sup>; Ajelli, M.<sup>1</sup>; Rizzo, C.<sup>2</sup>; Ciofi degli Atti, M.L.<sup>2</sup>

<sup>1</sup>Fondazione Bruno Kessler, Italy; <sup>2</sup>National Center for Epidemiology Surveillance and Health Promotion, Istituto Superiore di Sanita, Italy

Mathematical models have been used to describe the spatio temporal spread of a future pandemic and to examine the impact of some mitigation measures [1-3]. In general, while non pharmaceutical interventions can delay the epidemic arrival and the epidemic peak, pharmaceutical interventions can reduce the overall impact of the pandemic and the use of combined strategies guarantees the best results, especially when rapidly implemented [1-4]. In Italy, recent studies have shown that antiviral drugs,

used for treatment of cases and post-exposure prophylaxis of contacts, appear to be the most effective single intervention, resulting in an important reduction in cumulative attack rates (ARs), depending on the different hypothesized scenarios. Their use also contributes to delaying the peak day and to decreasing the peak daily AR [4]. Similar results have been shown in other European and U.S. studies [1,2]. However, the number of antiviral courses required for treatment and prophylaxis during a pandemic is extremely high. In Italy, in fact, they have been estimated at 10 million and 35 million courses, for  $R_0 = 1.7$  and  $R_0 = 2.1$ , respectively [4], out of a population of 57,000,000 inhabitants. Furthermore, it is unlikely that contact tracing for administering antiviral prophylaxis could be performed during the entire pandemic period. In this study we have thus considered different scenarios, for optimising the use of antiviral drugs. The worldwide spread of pandemic influenza and the consequent importation of cases in Italy were modelled using a global homogeneous-mixing SEIR (susceptible - exposed, but not yet infectious - infectious - recovered, and no longer susceptible) model (herein referred to as the "global SEIR model"). The national impact of an influenza pandemic in Italy and of various control measures was predicted using a stochastic individual-based SEIR model (IBM) [2,4]. In the global SEIR model, we assumed that infectious individuals were all symptomatic and no longer travelling and that exposed individuals were asymptomatic and possibly travelling before the infectious phase. To estimate the number of imported cases we coupled the results of the global SEIR model with 2003 data on arrivals and departures in Italy's 38 international airports. In the IBM, individuals were randomly grouped in households to match the 2001 census data on age structure and on household size and composition. Nine different types of households were considered (e.g., singles or couples, with or without children, with or without additional members, adults living together) and individuals were co-located in households according to specific data on the percentage of the different household types, their size, and the age of the household head. Children and young adults were assigned to one of six levels of school (i.e., from day care to university). Each working individual was randomly assigned to one of seven employment categories, defined by the number of employees in the workplace. We modelled travel destinations using data on commuting. Specifically, we employed a gravity model [5], in which the probability of commuting from one municipality to another increases with the population sizes and decreases with the distance. In both models, we assumed that the latency period for influenza was the same as the incubation period: duration of 1.5 ( $\pm 0.5$  SD) days. In the IBM, we assumed that the duration of infectiousness varied over time, as a lognormal function [2,6]. Infectiousness peaked at 1.75 days, and its duration was truncated at 10 days. This corresponded to an average generation time of 2.6 days. In the global SEIR model, the infectious period was assumed to be constant over time and was set at 1.5 days, to give essentially the same growth rate as the IBM [2,4]. In both the SEIR and IBM models, we considered different transmission rates to obtain  $R_0$  values of 1.4, 1.7, and 2,

which in the IBM corresponded to cumulative clinical AR of 21%, 30%, and 35%, respectively, indicating a mild, moderate and severe scenario. We took into consideration the administration of one course of antiviral drugs, providing treatment for the index case and prophylaxis for close contacts, i.e. household contacts. Both treatment and prophylaxis were assumed to start 24 or 48 hours after clinical onset in the index case. The treatment of the index case was assumed to reduce infectiousness by 70% [1,2,3,6], whereas AVP was assumed to reduce susceptibility to infection by 30%, infectiousness by 70%, and the occurrence of symptomatic disease by 60% [1]. We assumed that antiviral treatment is provided to 90% of the clinical cases (50% of all infected individuals) and that antiviral prophylaxis is provided to the close contacts of the treated index cases, with a treatment course of 10 days [7]. We considered administering antiviral treatment of index cases and prophylaxis for close contacts for the entire epidemic period. Population was divided into three main categories on the basis of the different clinical AR by age as resulting from the baseline simulations: children and young adults (2-25 years old), adults (26-64 years old) and elderly ( $\geq 65$  years old). We searched for the categories or for the combination of categories that allow for mitigating the epidemic by minimizing the number of antiviral courses required. To achieve such an aim, we consider the number of avoided clinical cases for each antiviral course as an indicator. Results are reported in Fig. 1. In the baseline simulations, the clinical AR is 21%, 29.5% and 35% in the three scenarios considered. If antivirals are used for treatment only, for all age groups, ARs will decrease to 11.6%, 21.7% and 28.8% requiring a number of antiviral courses of 5, 9 and 11.5 million (corresponding to 8.7%, 15.7% and 20.1% of the population), respectively. The number of avoided cases divided by antiviral courses used is 1.09, 0.49 and 0.31, respectively. When treatment is coupled to prophylaxis, the ARs are further decreased (4.6%, 13.6% and 19.9%), but a larger number of antiviral courses is required (7, 20 and 28.3 million courses, corresponding to the 12.4%, 35.4% and 49.7% of the population). The number of avoided cases for each antiviral course is similar to that observed with antiviral treatment only, namely 1.33, 0.45 and 0.3 for the three scenarios considered. Similar results are observed when treatment and prophylaxis are limited to individuals  $< 65$  years of age. Treatment for all individuals coupled to prophylaxis for younger individuals is the only strategy allowing a reduction of the AR (7.9%, 18%, 25.1%) with a significant reduction of antiviral courses (6.3, 13.7 and 18 million courses, corresponding to 11.1%, 24% and 31.6% of the population), at least in the mild and moderate scenarios. It is worth noticing that a delay of 2 days in both antiviral treatment and prophylaxis results in a dramatic decrease of the antiviral efficacy, namely about 50% in all the considered scenarios (see Fig. 2) and in general, while an increased number of antiviral courses results in a lower decrease of the AR. This means that preparedness for pandemic should not be only stockpiling of antiviral courses. In fact, a great effort should be made for organizing antiviral distribution and also for implementing specificity and sensitivity of existing surveillance

systems for seasonal influenza, in order to detect cases as soon as possible in the event of the emergence of an influenza pandemic.

Figure 1: Clinical attack rate, antiviral stockpile needed (% population) and number of avoid cases for each antiviral course by assuming different target categories for antiviral treatment of index cases and antiviral prophylaxis of household contacts under different scenarios. Delay in both treatment and prophylaxis is assumed to be 1 day.

Treatment			Prophylaxis			Clinical attack rate			Antiviral stockpile			# Avoided cases # Antiviral courses		
Age*			Age*			$R_0$			$R_0$			$R_0$		
Y	A	E	Y	A	E	1.4	1.7	2	1.4	1.7	2	1.4	1.7	2
✓	✓	✓	-	-	-	21.1	29.5	35.0	-	-	-	-	-	-
✓	✓	-	-	-	-	11.6	21.7	28.8	8.7	15.7	20.1	1.09	0.49	0.31
✓	✓	-	-	-	-	17.4	26.8	33.2	4.8	6.6	7.4	0.78	0.40	0.25
✓	✓	-	-	-	-	16.9	25.8	31.9	6.1	9.4	11.5	0.70	0.39	0.27
✓	✓	-	-	-	-	20.5	28.9	34.5	1.5	2.2	2.9	0.39	0.24	0.18
✓	✓	-	-	-	-	16.7	26.2	32.6	5.8	8.5	10.1	0.75	0.38	0.24
✓	✓	-	-	-	-	12.2	22.3	29.4	8.4	14.5	18.2	1.07	0.49	0.31
✓	✓	-	-	-	-	16.3	25.2	31.4	7.0	11.0	13.8	0.68	0.39	0.26
✓	✓	-	✓	✓	✓	4.6	13.6	19.9	12.4	35.4	49.7	1.33	0.45	0.30
✓	✓	-	✓	✓	✓	7.9	18.0	25.1	11.1	24.0	31.6	1.18	0.48	0.31
✓	✓	-	✓	✓	-	7.8	16.8	23.2	14.7	30.1	39.5	0.90	0.42	0.30
✓	✓	-	✓	✓	-	11.3	21.3	28.2	9.8	17.9	23.2	1.00	0.46	0.29
✓	✓	-	✓	✓	-	7.7	17.6	24.6	11.7	25.7	34.2	1.14	0.46	0.30
✓	✓	-	✓	✓	-	4.7	13.8	20.2	12.1	34.3	47.9	1.35	0.46	0.31
✓	✓	-	✓	✓	-	7.7	16.5	22.8	15.2	31.6	41.7	0.88	0.41	0.29
✓	✓	-	✓	✓	-	12.4	22.0	28.5	18.2	30.8	38.0	0.48	0.24	0.17
✓	✓	-	✓	✓	-	13.4	23.2	29.7	17.9	29.2	35.4	0.43	0.21	0.15
✓	✓	-	✓	✓	-	5.1	14.4	20.8	13.2	35.4	48.9	1.22	0.43	0.29

Figure 2: Clinical attack rate, antiviral stockpile needed (% population) and number of avoided cases for each antiviral course by assuming different target categories for antiviral treatment of index cases and antiviral prophylaxis of household contacts under different scenarios. Delay in both treatment and prophylaxis is assumed to be 2 days.

Treatment			Prophylaxis			Clinical attack rate			Antiviral stockpile			# Avoided cases # Antiviral courses		
Age*			Age*			$R_0$			$R_0$			$R_0$		
Y	A	E	Y	A	E	1.4	1.7	2	1.4	1.7	2	1.4	1.7	2
✓	✓	✓	-	-	-	21.1	29.5	35.0	-	-	-	-	-	-
✓	✓	-	-	-	-	14.8	24.2	30.6	11.7	18.6	23.0	0.54	0.29	0.19
✓	✓	-	-	-	-	18.7	27.7	33.7	5.4	7.2	8.2	0.45	0.24	0.16
✓	✓	-	-	-	-	18.0	26.7	32.7	7.2	10.8	13.2	0.43	0.25	0.17
✓	✓	-	-	-	-	20.6	29.0	34.6	1.6	2.5	3.2	0.29	0.18	0.13
✓	✓	-	-	-	-	18.2	27.2	33.3	6.7	9.4	11.1	0.43	0.24	0.16
✓	✓	-	-	-	-	15.2	24.6	31.1	11.0	17.0	20.7	0.54	0.28	0.19
✓	✓	-	-	-	-	17.5	26.3	32.3	8.3	12.7	15.9	0.43	0.25	0.17
✓	✓	-	✓	✓	✓	8.3	17.1	23.2	20.6	40.4	52.4	0.62	0.31	0.23
✓	✓	-	✓	✓	✓	11.6	21.1	27.7	15.8	27.2	34.0	0.61	0.31	0.21
✓	✓	-	✓	✓	-	11.3	19.9	25.9	19.9	33.4	41.6	0.50	0.29	0.22
✓	✓	-	✓	✓	-	14.5	23.8	30.2	12.9	20.8	25.9	0.51	0.27	0.19
✓	✓	-	✓	✓	-	11.3	20.8	27.3	16.7	29.1	36.6	0.59	0.30	0.21
✓	✓	-	✓	✓	-	8.5	17.3	23.5	20.1	39.1	50.4	0.63	0.31	0.23
✓	✓	-	✓	✓	-	11.1	19.7	25.6	20.6	35.1	43.9	0.49	0.28	0.21
✓	✓	-	✓	✓	-	14.7	23.9	30.0	21.1	32.7	39.4	0.31	0.17	0.13
✓	✓	-	✓	✓	-	15.5	24.8	31.0	20.5	31.0	36.9	0.27	0.15	0.11
✓	✓	-	✓	✓	-	8.8	17.8	24.0	20.9	40.0	51.2	0.59	0.29	0.22

\* Y: 2-25 years old, A: 26-64 years old, E: ≥ 65 years old.

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9-007

### Economic evaluation of influenza pandemic mitigation strategies in the US using a stochastic microsimulation influenza model

Sander, Beate<sup>1</sup>; Nizam, A.<sup>2</sup>; Garrison Jr., L.P.<sup>3</sup>; Postma, M.<sup>4</sup>; Halloran, M.E.<sup>3</sup>; Longini Jr., I.M.<sup>3</sup>

<sup>1</sup>University of Toronto, Canada; <sup>2</sup>Emory University, USA; <sup>3</sup>University of Washington, USA; <sup>4</sup>University of Groningen, Netherlands

**Purpose:** To project the potential impact of pandemic influenza mitigation strategies on health outcome, cost, and cost-effectiveness from a societal perspective.

**Methods:** We use a stochastic agent-based model to simulate the impact of pandemic influenza on a typical American community of 1.6 million. We compare 16 strategies to no intervention, focusing on targeted antiviral prophylaxis (TAP) with oseltamivir (treatment of identified index cases and prophylaxis of exposed people in the key mixing groups of the index case) alone and in combination with school closure. We assume three levels of antiviral stockpile would be available: 25% and 50% of the population, and unlimited. We also consider pre-vaccination of 70% of the population. We use the human capital approach to estimate productivity loss. Outcomes include number of cases, deaths, QALYs, direct and indirect costs, and incremental cost-effectiveness ratios (ICERs) expressed as costs per QALY gained.

**Results:** In the absence of intervention, we predict a 50% attack rate with an economic impact of \$187 per capita. TAP+school closure and pre-vaccination+school closure (preventing 94-96% of cases at \$2,730 per capita) are comparable in terms of QALY gain and total costs. The ICER compared to TAP alone (the most effective single strategy) is about \$50,500/QALY for either strategy. TAP alone (prophylaxis of 60% of close contacts of index cases) effectively prevents 54% of cases at a cost of \$120 per capita. If vaccine is available and administered before the onset of the pandemic, then pre-vaccinating 70% of the population with a partially effective vaccine prevents 48% of cases and is the least

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costly alternative (\$99 per capita). Sensitivity analysis on key variables does not change the ranking of strategies but shows that the basic reproductive number has the greatest impact on QALYs and hence ICERs.

**Conclusions:** All interventions reduce the illness attack rate, morbidity and mortality. Many interventions are also cost-saving compared to no intervention. Targeted antiviral prophylaxis is an effective and cost-saving measure for mitigating pandemic influenza. Adding school closure provides greater benefit and is likely to be an attractive strategy if transmission and mortality is high.

9-008

### Impact of the implementation of rest days in live bird markets on the dynamics of low and highly pathogenic avian influenza: a modelling approach

Mangtani, P.<sup>3</sup>; Ghani, A.C.<sup>2</sup>; Guitian, J.<sup>1</sup>

<sup>1</sup>Royal Veterinary College, UK; <sup>2</sup>MRC Centre for Outbreak Analysis & Modelling - Imperial College, UK; <sup>3</sup>London School of Hygiene and Tropical Medicine, UK

**Background:** Live bird markets (LBMs) are known to play a key role in the epidemiology of avian influenza viruses. Acting as a network “hub” and potential reservoir of infection for domestic poultry, they may be responsible for sustaining endemic infection within the poultry sector. Rest days, during which markets are emptied and disinfected, have been implemented in Hong Kong LBMs, resulting in a decrease in the rate of isolation of avian influenza virus in these markets. Here, we investigate the potential of rest days to modulate the dynamics of low and highly pathogenic avian influenza (LPAI and HPAI) within the poultry sector.

**Methods:** We use a stochastic metapopulation model to simulate the spread of LPAI and HPAI within the poultry sector. We consider a vertical market system with a unidirectional flow of poultry: from farms, poultry are stored in a unique wholesale market before being transferred to the retail markets. The infection can spread by commercial poultry movements across the market system, or by indirect contacts between farms or between farms and retail markets. For HPAI, we assumed a relatively high  $R_0=40$  within the farm and  $R_0=10$  within the regional and wholesale markets and an infectious period of 2 days. For LPAI we assumed a lower  $R_0=2$  within the farms and the markets and an infectious period of 4 days. For HPAI, stamping out is also applied in the infected premises when the mortality rate exceeds 0.5%. Rest days are implemented simultaneously in the wholesale and regional markets and we assume optimistically that these completely remove the infectious reservoir. We use the model to assess the impact of the frequency of rest days on the proportion of farms

experiencing LPAI/HPAI infection.

**Results:** Compared to a market system without rest days, implementation of a weekly rest day which occurs simultaneously in the wholesale and the retail markets has a strong impact on the dynamics of HPAI infection, reducing the mean proportion of infected premises by approximately 60%. However, the efficiency of this intervention drops dramatically as the time between two successive rest days increases. If the frequency of rest days is only two or one per month, the mean proportion of infected farms decreases by only 25% and 12% respectively. In contrast, this intervention has relatively little impact on LPAI infection with the implementation of a rest day every week resulting in only a minor decrease in the proportion of infected premises (by less than 5%) due to the lower  $R_0$  assumed for LPAI. Our results are also sensitive to the degree to which indirect transmission occurs directly between farms, and between farms and the regional markets.

**Conclusion:** Frequent rest days in live bird markets are an efficient means with which to reduce HPAI prevalence. However, their impact will depend on the patterns of contacts between farms, regional markets and wholesale markets as well as the risk of transmission between each of these locations. Further data on the different market systems in AI-affected countries could help to elucidate the potential impact of rest days as well as to define their optimal frequency in terms of their impact on circulating HPAI and LPAI viruses.

9-009



### Extrinsic and intrinsic determinants of influenza seasonality in temperate and tropic zones

Meeyai, A.; Ferguson, N.M.

Imperial College (London), UK

The causes of seasonal fluctuations in influenza incidence remain obscure. Recently, Dushoff *et al.* showed that it may be impossible to establish the underlying cause of seasonality in influenza epidemics because the large observed oscillations in incidence could be generated by undetectably small seasonal changes in the transmission rate as a result of resonant effects between extrinsic forcing (resulting from effects of seasonally-varying environmental and/or behavioural factors) and the intrinsic periodicity of the dynamic system.

In order to evaluate the extent to which extrinsic and intrinsic determinants of seasonality may explain observed patterns of influenza incidence in both temperate zones and the tropics, the mechanistic TSIR (time-series susceptible infected recovered) model was applied to: A) simulated influenza data under

conditions of both strong and weak resonance; B) influenza-like illness (ILI) weekly incidence data from the United Kingdom (UK) and France from 1984 to 2004; and C) ILI weekly incidence data from Thailand, 1988 to 2004, and weekly ILI data from Hong Kong, 1998 to 2006.

Using data simulated from a stochastic compartmental transmission dynamic model of influenza and the discrete TSIR model under conditions of both strong and weak resonant effects, it was found that, surprisingly, the TSIR model was able to accurately estimate the extrinsic seasonal component of transmission and distinguish it from the intrinsic determinants. This was the case even when the extrinsic seasonal forcing was extremely small. When applied to real data, it was found that the very large annual fluctuations in influenza incidence in the UK and France and the smaller annual fluctuation in Thailand and Hong Kong could be explained by a detectable exogenous seasonal component of influenza transmission, rather than an undetectably small change in the transmission rate amplified by population dynamic resonance of the host-pathogen system. However, analysis of simulated data suggested that when departures from the model assumptions of homogeneous mixing were allowed, estimates of extrinsic seasonal components of variation could be sensitive to the precise data generation process.

As with any passive surveillance systems, under-reporting of ILI cases is a concern. Conversely, not all patients identified by their GPs as having ILI will actually be infected with an influenza virus. Initial results suggest that the broad conclusions will not be affected by under-reporting in the surveillance data. Future work will explore the effects of variations in the reporting of ILI between age groups.

9-010

### Estimates of the $R_0$ of the 1968 (Hong Kong) influenza pandemic: evidence for increased transmissibility of pandemic influenza between successive waves

Jackson, Charlotte<sup>1</sup>; Vynnycky, E.<sup>1</sup>; Mangtani, P.<sup>2</sup>

<sup>1</sup>Health Protection Agency, UK; <sup>2</sup>London School of Hygiene and Tropical Medicine, UK

**Background:** The transmissibility of the strain which caused the 1968 influenza pandemic is poorly understood. It may even have increased between successive waves, given increases in the outbreak sizes between the first and second waves. Elucidation of such increases is important for planning interventions for future pandemics.

**Methods:** Epidemic curves and overall attack rates for the 1968 influenza pandemic, based on clinical and serological data, were retrieved from published literature for 41 and 16 settings for the

first and second waves respectively of the 1968 pandemic. The basic and net reproduction numbers of the virus were estimated for each dataset based on the growth rate and / or the final size of the epidemic.

**Results:** The basic reproduction number was estimated as ~1.2 during the first wave and ~1.2-3.5 during the second. Within each wave, there was little geographic variation in transmissibility: for example,  $R_0$  was estimated as 1.10-2.00 in 8 British settings and as 1.18-1.70 in 4 Australian settings during the first wave. In all 10 settings where data were available for both waves,  $R_0$  was estimated to be higher during the second wave than the first (e.g. 1.54 and 2.77 during the first and second waves, respectively, in Lambeth, England).

**Conclusions:** Based on documented clinical and serological attack rates it appears that the larger outbreaks seen during the second wave of the 1968 H3N2 pandemic, as compared with the first wave, may have been partly due to an increase in transmissibility. This potential for change in viral behaviour may have consequences for future pandemic mitigation strategies.

9-011

### Revealing associations between H5N1 mutations using Bayesian Graphical Models

Lycett, S.J.<sup>1</sup>; Ward, M.J.<sup>1</sup>; Lewis, F.I.<sup>2</sup>; Poon, A.F.Y.<sup>3</sup>; Kosakovsky Pond, S.L.<sup>3</sup>; Leigh-Brown, A.J.<sup>1</sup>

<sup>1</sup>University of Edinburgh, UK; <sup>2</sup>Veterinary Epidemiology Research Unit, SAC, Inverness, UK; <sup>3</sup>University of California San Diego, USA

The highly pathogenic avian influenza (HPAI) virus H5N1 has been a cause for concern since its recent emergence in Asia in 1996 due to its pathogenicity in water and land fowl and its cross species transmissibility and pathogenicity to other mammals including humans (Li 2004). During the course of this epidemic in birds, the virulence of circulating H5N1 has increased through mutations (Chen 2004) and it has spread into Europe, Africa and America. The aim of this paper is to show how Bayesian Graphical Models can be used to elucidate the associations between mutations in H5N1 sequences from avian hosts, and how these mutation patterns might be related to host type and virulence.

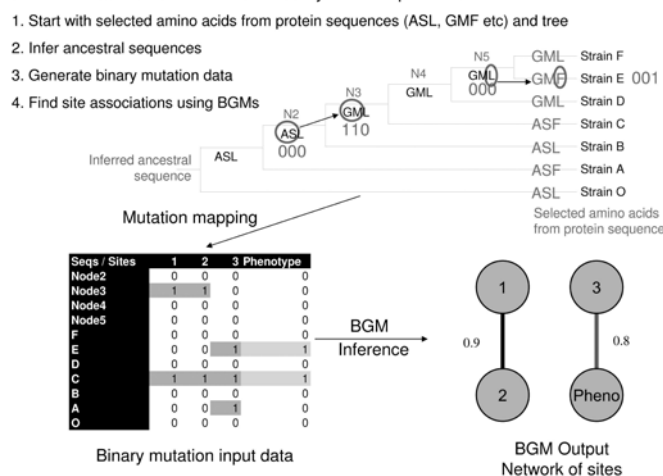
A Bayesian Graphical Model consists of nodes connected by edges representing conditional dependencies. In this case the nodes are the variable (mutation) sites in the 8 segments of the H5N1 genome. To infer the probabilistic dependencies between the nodes, the sequences are pre-processed into binary form by coding each variable site per sequence as a one if the site is different to a reference or ancestral sequence, or a zero if it is the same. By using ancestral sequences inferred from the data and the phylogenetic trees (one per segment) as

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references, the resulting binary data includes the evolutionary history of the sequences (Poon 2007). Next, the conditional dependencies between the variable sites are estimated using a Monte Carlo Markov Chain technique to sample from appropriate graph structures and parameters (Friedman & Koller 2003). An additional response variable node for host type (0 if water fowl or 1 if terrestrial (eg chickens)) was also included in the analysis to enable associations between patterns of sites and the host type to be inferred.

This Bayesian Graphical Modelling approach identified some interesting associations between sites on different segments, including an HA glycosylation site and an NA site. Using the ancestral reconstruction binary data, several patterns of within gene site association were found, and some mutations in HA, NA and NP were directly associated to host type. To verify that the Bayesian Graphical Model approach can reveal real associations between nodes using only binary data, we also simulated binary data from representative graphs and measured the errors in the inferred graphical models as a function of the number of nodes, relationship between the nodes, and number of sequences simulated.

### Amino acid mutation association with Bayesian Graphical Models



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9-012

### Geo-temporal spread of influenza A in Canada with a comparison to patterns for the United States and Europe

Schanzer, Dena<sup>1</sup>; Dummer, T.<sup>2</sup>; Langley, J.M.<sup>2</sup>; Aziz, A.<sup>1</sup>; Viboud, C.<sup>3</sup>; Winchester, B.<sup>1</sup>; Tam, T.W.S.<sup>1</sup>

<sup>1</sup>Public Health Agency of Canada, Canada; <sup>2</sup>IWK Health Centre, Dalhousie University, Canada; <sup>3</sup>Fogarty International Center, NIH, USA

**Introduction:** A better understanding of the spread and control of annual influenza epidemics is considered key to pandemic planning. We used Canadian data to analyze the geo-temporal spread at varying geographic scales and compared the Canadian experience with American and European data.

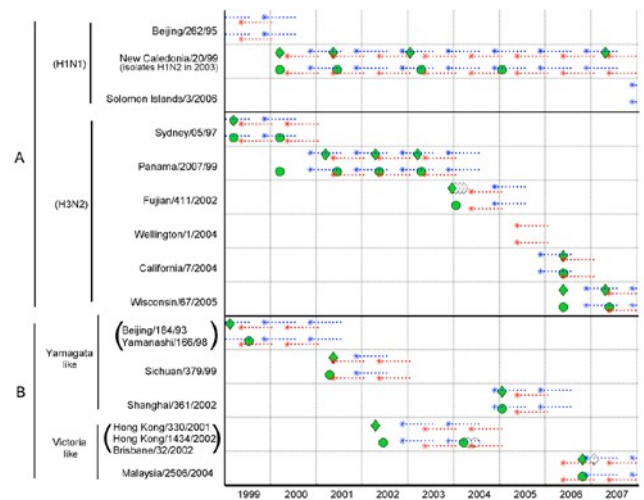
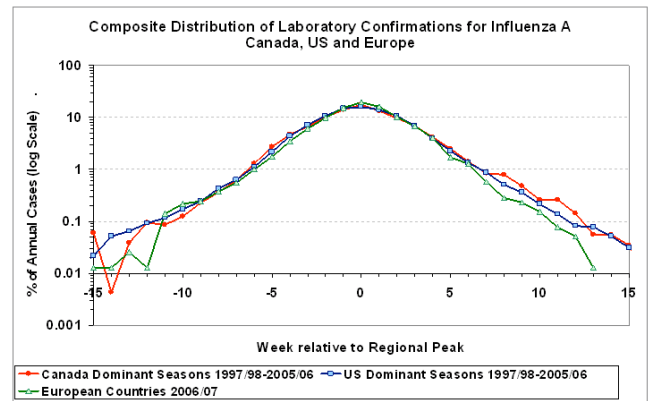
**Methods:** Weekly Canadian laboratory viral identifications (FluWatch, PHAC) for influenza A and respiratory hospital admissions from the 1997/98-2005/06 seasons were augmented with weekly laboratory confirmations for influenza from the United States CDC Flu surveillance program and with European data for the 2006/07 season provided by the WHO. We identified the timing of the local epidemic by the week representing the midpoint of influenza A laboratory confirmations, and the peak week for respiratory hospital admissions from the Canadian hospitalization database. The geographic unit varied from the city level to provincial or regional levels. A composite epidemic curve was created by centering the local epidemics relative to their epidemic mid-point and aggregating the weekly number of confirmations for various geographic units or seasons. The weekly epidemic growth rates, estimated using Poisson regression, as well as differences in regional timing were compared across regions or seasons.

**Results:** The influenza epidemic was similar in timing and shape for urban and rural areas in Canada. Influenza activity was on average 3 weeks later in the Atlantic Provinces than for central and western provinces and the United States. Peak influenza A activity generally spread across central and western Canada and the United States over the same 6 to 13 weeks. There was considerable variability in the direction of spread. The epidemic was often closely synchronized in adjacent regions, though these same regions could see a delay of up to 5 weeks in other years (milder, H1N1 seasons). The composite epidemic curve suggests that the dominant strain emerges approximately 10 weeks prior to peak activity and that the exponential growth continues at approximately the same rate until a couple of weeks before peaking. Influenza A spreads faster within a community than influenza B. The epidemic growth rate for influenza A was similar for Canada, US and Europe: it takes approximately 1 month for the number of weekly cases to increase 10-fold. The estimated weekly epidemic growth rate was similar for most subgroups (urban versus rural and city size; province, US region; H1N1 vs. H3N2 seasons; month of peak), ranging from 1.5 to 2.0. Further analysis will assess whether a consistent pattern will emerge

from any of the statistically significant differences. For Canada, US and Europe, at the aggregate level, the average weekly epidemic growth rate for influenza A ranged from 1.7 to 1.8 and differences were not statistically significant.

**Conclusions:** Despite wide variation in climate and the environment, we generally did not detect a consistent pattern in the timing of the epidemic across large geographic regions, though rates of transmission within a community seemed consistent. Both weekly laboratory confirmations of influenza and respiratory admissions suggest that influenza cases appear to be geographically widespread early in the epidemic and that the timing and spread of influenza within urban and neighbouring rural areas was similar. However, at the smaller scale of cities and towns, there is evidence of fairly stable transmission patterns. Approximately 10 weeks before the epidemic peak in seasons with a dominant influenza A strain (>80% of isolates), the dominant strain started to emerge, at least in larger urban centers. This is very early in the epidemic, and most surveillance methods are not likely to be able to detect excess influenza cases until approximately 5 weeks later.

Variation in the direction of spread suggests that it is unlikely that travel alone is responsible for the dynamics of influenza transmission. As influenza appears to be geographically widespread early in the epidemic, the annual epidemic likely starts independently in multiple regions. The annual epidemic starts in the fall though peaks in winter.



9-013



### The effects of influenza vaccination of institutional health care workers: insights from a mathematical model

van den Dool, Carline<sup>1</sup>; Bonten, M.J.M.<sup>1</sup>; Hak, E.<sup>1</sup>; Heijne, J.C.M.<sup>2</sup>; Wallinga, J.<sup>2</sup>

<sup>1</sup>UMC Utrecht, Netherlands; <sup>2</sup>National Institute for Public Health and the Environment, Netherlands

**Background:** Most Western countries advise annual influenza vaccination of institutional health care workers (HCWs), but adherence to the recommendation is generally low. The protective effects of HCW vaccination for long-term care patients have been demonstrated in some clinical trials, but the exact relationship between increased vaccine uptake among HCWs and protection of patients remains unknown due to variations between study designs, settings and intensity of influenza seasons. Instead of conducting a very large clinical trial to control for all possible effect modifiers, we use a mathematical model to study the effect of increased HCW vaccination and the potential existence of a herd immunity threshold in nursing home and hospital departments.

**Methods:** We use a stochastic individual-based model with discrete time intervals to simulate influenza virus transmission in a long-term care nursing home department and a short-term care hospital department. We simulate different levels of HCW vaccine uptake and study the effect on influenza virus attack rates among patients for different institutional and seasonal scenarios.

**Results:** Our model reveals a robust linear relationship between the number of HCWs vaccinated and the expected number of influenza virus infections among nursing home patients. No threshold for herd immunity can be detected. In a standardized nursing home department, approximately 60% of influenza virus infections among patients can be prevented when the HCW vaccination rate increases from 0 to 1. Due to stochastic variations, the differences in patient attack rates between departments are high and large outbreaks can occur for every level of HCW vaccine uptake. The results for hospital departments are now being generated and will be presented at the conference.

**Conclusions:** Due to the absence of herd immunity in nursing homes, every additional HCW vaccination leads to the protection of an additional fraction of patients. Large stochastic variations between departments suggest that the results of small-sized clinical trials on the effects of HCW vaccination should be interpreted with great care. A power calculation based on the likely size of these variations indicates that all studies performed so far should be considered underpowered to determine the precise effects of HCW vaccination. This finding should be taken into account when designing future studies.

9-014



### A model for estimating influenza vaccine impact in England & Wales

Mann, A.<sup>1</sup>; Mangtani, P.<sup>1</sup>; Russell, C.A.<sup>2</sup>; Whittaker, J.C.<sup>1</sup>

<sup>1</sup>London School of Hygiene & Tropical Medicine, UK; <sup>2</sup>University of Cambridge, UK

One way to estimate the unknown impact of influenza vaccination in England & Wales is to isolate the long-term trend in morbidity or mortality attributable to influenza and express this trend as dependent on vaccination coverage. Previous work has assumed that this trend is linear and that weekly counts come from a single distribution. The epidemiology of influenza, where normal seasonal activity is punctuated by irregular large epidemics, suggests that neither assumption is ideal. We fitted age-group-specific Poisson log-linear models allowing for seasonality and overdispersion to weekly sentinel general practice consultations for influenza-like illness (1967-2005), deaths from pneumonia or influenza (1970-2005) and influenza laboratory reports (1975-2005). In order to isolate the long-term trend, we excluded weeks in the top 1% for each outcome. Modeling trend in a flexible manner, with quadratic and cubic spline terms, improved model fit and showed that the assumption of a linear trend does not hold for these data. Trend also differed by age group. The exclusion of 1% of counts was insufficient to remove the effect of large epidemics. We will model the effect of large epidemic peaks using a two-state hidden Markov model with cubic splines to better reflect the epidemiology of influenza. Here, unusually large counts are drawn from one probability distribution, and normal counts from another, while trend need not be linear. We will estimate vaccination impact by expressing long-term trend or the height of large epidemics as dependent on vaccine coverage. We will also explore extensions to this model allowing for interaction between antigenic drift in influenza A/H3N2 and vaccine impact.



## How well have the influenza vaccination campaigns in the Southern Hemisphere placed their bets? A tale of two distant cities in Brazil

Mello, W.A.<sup>1</sup>; Paiva, T.M.<sup>2</sup>; Ishida, M.A.<sup>2</sup>; Benega, M.A.<sup>2</sup>; Santos, M.C.<sup>3</sup>; Viboud, C.<sup>4</sup>; Miller, M.<sup>4</sup>; **Alonso, W.J.**<sup>4</sup>

<sup>1</sup>Evandro Chagas Institute (IEC) / WHO Global Influenza Surveillance Network (GISN) / Secretary of Surveillance in Health / Brazil, Brazil; <sup>2</sup>Adolfo Lutz Institute (IAL), WHO Global Influenza Surveillance Network (GISN), and Brazilian Ministry of Health, São Paulo, SP, Brazil; <sup>3</sup>Evandro Chagas Institute (IEC) / WHO Global Influenza Surveillance Network (GISN) / Secretary of Surveillance in Health, Brazil; <sup>4</sup>Fogarty International Center - National Institutes of Health, United States

**Background:** Given the rapid antigenic evolution of the influenza virus and global circulation of strains, the World Health Organization issues two recommendations for influenza vaccine composition each year, prior to the influenza season in Northern and Southern hemispheres. These recommendations take into account the most likely circulating strains in the next influenza season and time needed for vaccine production and delivery. Vaccine effectiveness varies each season and depends on how well the vaccine strains match the circulating strains, as well as whether serum antibody response has peaked in the target population before the epidemic arrives. Influenza epidemics display weak seasonality in Tropical regions, which have intermediate geographical position between temperate countries in both Hemispheres, creating additional challenges to the correct matching of the vaccine timing and composition. Here we quantify the effectiveness of the influenza vaccination strategy adopted in Brazil and compare this strategy with alternative ones, considering different timings of vaccination and strain compositions.

**Methodology:** We relied on monthly data on influenza virus circulation and strain characterization collected in two Brazilian cities, Belém, located at the Equator, and São Paulo, at the limit between the tropical and subtropical regions, between 1999 and 2007. We estimated the proportion of seasons with correct vaccine match, defined by the temporal overlap between vaccine-induced protection and circulating strains, separately for each influenza (sub)type. We considered two possibilities for vaccine-induced protection duration: 9 or 12 months. We then estimated the proportion of correct vaccine matches considering that vaccination campaigns were generally conducted during the second half of April and first week of May of each year (for the sake of simplicity we considered that protective vaccine response was achieved by early May) and relied on the Southern Hemisphere composition -- the strategy historically adopted in Brazil since 1999. Second, we estimated

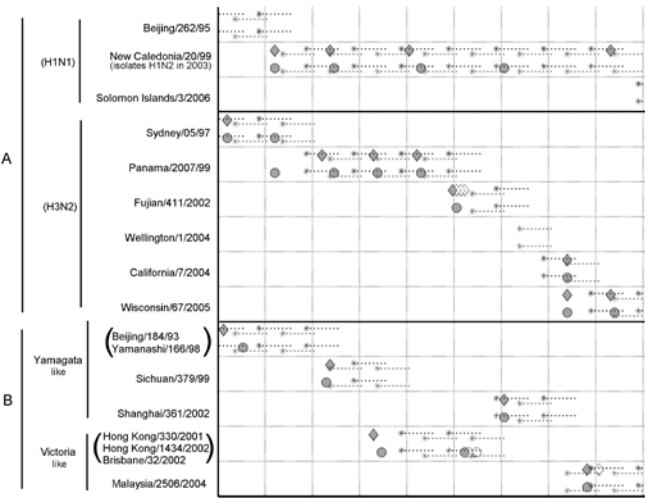
vaccine match for alternative strategies, relying on the Northern Hemisphere vaccine recommendations and/or earlier timings of vaccination (also assuming that protective vaccine response was achieved in the month following the vaccination campaign month).

**Principal Findings:** Analyses assuming that vaccine protection lasted for 9 months showed more dramatic results than analyses assuming a 12-month protection (table 1), given that a specific influenza strain often keeps circulating for several consecutive seasons. Focusing on a vaccine-induced protection of 9 months, we conclude that delivery of the Southern Hemisphere vaccine in January rather than April would have more than doubled (from 30% to 70%) the proportion of correct vaccine matches with observed influenza epidemics (Table 1; chi-square,  $p < .01$ ). Surprisingly, a strategy relying on the composition and timing defined by the Northern Hemisphere recommendations would have also doubled (from 30% to 65%) the matching proportion (Figure 1; Table 1; chi-square,  $p < .01$ ). This finding was consistent for both the equatorial and subtropical Brazilian cities, and for all 3 influenza (sub)types, although weaker in H1N1 group given its lower rate of antigenic change during this period.

**Conclusion:** Influenza vaccine recommendations and timing of vaccine delivery have to be tailored to the specific epidemiological situation of each location. Given the complexities inherent to the circulation of influenza in the Tropics, our results suggest that the Southern Hemisphere recommendations are too late for Brazil, and vaccine is delivered while the epidemiologic season is in an advanced stage. For Brazil, correct timing of vaccination seems the most important factor for vaccine success. Therefore, our results suggest that --under the current impossibility of the ideal scenario of delivering the Southern Hemisphere recommendation earlier-- the adoption of the vaccine recommendations designed for the Northern Hemisphere may be more appropriate for the Brazil. This situation may be generalized to other tropical regions of the Southern Hemisphere, an area encompassing the majority of the population in this Hemisphere. Further studies in other Tropical regions of Africa, Asia and Oceania are warranted. Figure 1) Matching success of vaccine strategies, against strains of influenza viruses isolated monthly from 1999 to 2007 in Belém and São Paulo, assuming a 9-month vaccine-induced protection. The different categories of influenza strains considered in the study period are indicated on the vertical axis, sorted by influenza subtype (influenza A) and lineage (influenza B) and identification date. Time is measured on the horizontal axis. Strains isolated each month are represented by green diamonds for Belém, and green circles for São Paulo. Stars represent the first month of this period, while the following dotted lines represent the remaining months. Red lines correspond to historic vaccination strategy adopted by Brazilian authorities, relying on the Southern Hemisphere vaccine recommendations and schedule. Blue lines represent a hypothetical scenario where the Northern Hemisphere

vaccination recommendations and schedule are used in both cities. Rate of successful matches between vaccines and circulating strain is quantified by the overlap between vaccine data and actual virus isolations through this period. Table 1: Success of vaccination campaigns in Brazil (assuming 9 and 12 months of protection) for the historical strategy and simulated alternative strategies for the period 1999-2007. Cells indicate the number and proportion of seasons where influenza strains isolated in São Paulo and Belém were correctly matched with each vaccination strategy, taking into account the match between circulating strains and vaccine composition, and the timing of the vaccination campaign. The total number of strains identified in each location is indicated in the headers (without repetition in the same season, influenza subtype, year and place).

	12-month protection	12-month protection	12-month protection	9-months protection	9-months protection	9-months protection
Vaccination Scenarios	Belém (n=15)	São Paulo (n=18)	Overall (n=33)	Belém (n=17)	São Paulo (n=20)	Overall (n=37)
Southern formulation, delivered following Southern schedule (April)	8 (53%)	11 (61%)	19 (58%)	5 (29%)	6 (30%)	11 (30%)
Northern formulation, delivered following Southern schedule (April)	6 (40%)	9 (50%)	15 (45%)	3 (18%)	7 (35%)	10 (27%)
Southern formulation, delivered earlier than Southern schedule (January)	11 (73%)	13 (72%)	24 (73%)	12 (71%)	14 (70%)	26 (70%)
Northern formulation, delivered following Northern schedule (October)	9 (60%)	11 (61%)	20 (61%)	11 (65%)	13 (65%)	24 (65%)



9-016

Revisiting the classical W-shape of 1918 pandemic influenza mortality: The true meaning of catastrophe

Simonsen, Lone<sup>1</sup>; Andreassen, V.<sup>2</sup>; Olson, D.R.<sup>3</sup>; Molbak, K.<sup>4</sup>; Viboud, C.<sup>5</sup>

<sup>1</sup>George Washington University, USA; <sup>2</sup>Roskilde University, Denmark; <sup>3</sup>International Society for Disease Surveillance, USA; <sup>4</sup>Statens Serum Institute, Denmark; <sup>5</sup>Fogarty International Center, NIH, USA

**Introduction:** Recent studies [1,2] using high resolution mortality data from New York City (NYC) and Copenhagen have challenged the traditional view that the age-specific mortality pattern in the 1918-19 Spanish flu-pandemic was “W-shaped”, with highest risk in infants, young adults and seniors. In contrast, these recent studies have uncovered a complete mortality sparing among seniors, while confirming a sharply elevated risk in young adults. Here we continue the effort to revisit the “W-shape”, using historical data from a unique surveillance system in Copenhagen, “Ugelisterne”, where all physicians reported weekly on medically attended illnesses, hospitalizations and deaths, by cause and age. Based on these data, we quantify how “catastrophic” the pandemic was across the entire age spectrum, including 3 pediatric age groups, and explore the risk of pandemic death relative to background mortality. We also compare age patterns of “catastrophe” in Copenhagen [2] with that in NYC [1].

**Methods:** For Copenhagen we accessed incidence of respiratory illness, respiratory deaths, and all-cause deaths for 1910-1921, by week and age. For NYC we extracted pneumonia and influenza (P&I) deaths for 1910-1920, by week and age. The elevation in deaths during the influenza period, measured over a model baseline of deaths, was considered attributable to influenza (excess mortality). In this study we found that a traditional Serfling model baseline produced negative rates of excess mortality for 1918 in senior age groups. Therefore, we instead computed the ratio of mortality rate in the severe fall wave, Oct-Dec 1918, to the average “background” mortality rate during Oct-Dec 1910-1917, for each age group and city. The resulting age-specific relative risk (RR) of death associated with the 1918 pandemic was plotted and contrasted with the classical “W-shaped” 1918 mortality curve.

**Results:** Weekly mortality data from Copenhagen and NYC demonstrated the well-established extreme 1918 pandemic risk elevation in young adults, and confirmed the findings of complete sparing of seniors [1,2]. When we computed the relative risk (RR) of pandemic mortality relative to background, the “W” gave way to an “Opposite V shape”: Infants <1 and toddlers 1-4 years of age were at no greater risk of dying during the pandemic than they were in previous years (RR~1.0). Children 5-17 years old had some risk elevation, but the risk peaked in young adults (RR=6 for all-cause- and RR=20 for respiratory deaths), then declined after age 45. Seniors, like young children, were at no greater risk of dying during the pandemic than in previous years

(RR~1.0). In summary, neither pediatric nor senior age groups experienced a “Catastrophic” pandemic impact. In contrast, all age groups, except seniors, had dramatically elevated levels of medically attended respiratory illness during the 1918 pandemic in Copenhagen.

**Conclusions:** The true meaning of “Pandemic Catastrophe” takes on new meaning when considering the relative mortality risk increase over the expected “background” risk of dying. The lack of relative risk increase in extreme age groups has important implications for contemporary pandemic planning which focuses on a 1918-like “worst case” scenario [3]). When it comes to prioritizing age groups for a likely scarce pandemic vaccine supply [4], most countries plan to do “influenza business as usual” and vaccinate seniors before other age groups. Instead, the “relative risk elevation” point of view presented here would prioritize middle age groups truly experiencing “Catastrophe”. For a future pandemic scenario of an A/H5N1 virus or other zoonotic influenza virus threats, it cannot be known in advance which age groups will be at highest risk elevation. It is therefore critical to compile and analyze real time data on age mortality patterns from multiple locations, during the first wave of a future pandemic. With flexible vaccine priority guidelines and timely information on pandemic mortality impact, age groups experiencing “Catastrophe” can be targeted and vaccine benefits optimized.

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## 10 IMMUNOLOGY

10-001

### NF-κB and ERK inhibition reduces expression of pro-inflammatory factors and virus propagation simultaneously in influenza virus-infected mice

Pinto, R.<sup>1</sup>; Herold, S.<sup>2</sup>; Cakarova, L.<sup>2</sup>; Lohmeyer, J.<sup>2</sup>; Seeger, W.<sup>2</sup>; Pleschka, S.<sup>1</sup>

<sup>1</sup>Institute for Medical Virology, University of Giessen, Germany; <sup>2</sup>University Giessen Lung Center, Dept. Internal Medicine, Giessen, Germany

Influenza virus (IV) infection can cause severe pneumonia and lead to acute respiratory distress syndrome. Despite damage caused by viral replication, unbalanced inflammation can also lead to severe lung damage. Therapeutics such as vaccines and anti-viral drugs target components of the virus itself resulting in resistant variants. Therefore new therapeutic measures are urgently needed. We have previously shown that IV activates the MAPK- and the NF-κB signalling pathway. These pathways seem to have both pro- and anti-viral effects, by promoting nuclear RNP export and by inducing expression of anti-viral pro-inflammatory factors. Using specific MEK- and IKK-inhibitors we analyzed the effect of MAPK- and NF-κB pathway inhibition on virus propagation and cytokine induction in vitro in IV-infected human lung epithelial cells and mice primary alveolar epithelial cells as well as in vivo by analyzing lung homogenates and BAL of influenza-infected mice. Our results demonstrate that: inhibition of MAPK- and NF-κB pathway can simultaneously reduce virus titres and modulate pro-inflammatory cytokine expression in vitro as well as in vivo. This could be important for future therapeutic strategies to treat IV pneumonia.

10-002

### The HA2 glycopolypeptide of influenza A virus haemagglutinin induces a protective immune response against influenza A infection of mice

Janulikova, J.<sup>1</sup>; Gocnik, M.<sup>2</sup>; Fislova, T.<sup>2</sup>; Stanekova, Z.<sup>2</sup>; Mucha, V.<sup>2</sup>; Kostolansky, F.<sup>2</sup>; Vareckova, E.<sup>2</sup>

<sup>1</sup>Institute of Virology, SAS, Slovakia; <sup>2</sup>Institute of Virology, SAS, Bratislava, Slovakia

A new approach to “universal” vaccine preparation is based on looking for regions of influenza virus proteins inducing cross-protective immunity. HA2 gp, the conserved part of haemagglutinin

(HA), fulfils all requirements to be one of the potential inducers of heterosubtypic immunity. It reveals high inter-subtype sequential homology [5, 6, 9] and is a good immunogen. HA2 gp induces a complete immune response. The level of HA2-specific antibodies increases significantly after the repeated natural infection [4]. The biological function of HA2-specific antibodies was not clear until we showed that some HA2-specific monoclonal antibodies (MAb) inhibited the fusion activity of HA and reduced in vitro and in vivo virus replication [2, 3, 7, 8]. Therefore the aim of the presented work was to find out whether HA2 gp is able to induce protective immune response in mice against influenza infection. As an immunogen we used purified EHA2 construct (23-185 aa of HA2) expressed by transformed *E. coli* [1] originating from A/Aichi/1/68 (H3N2) virus. BALB/c mice were immunized intraperitoneally with two consecutive doses of HA2 gp together with complete or incomplete Freund adjuvants (FA) in 14-day intervals. Control groups of mice received FA without EHA2 in the same intervals. Two weeks after the second immunization, mice were infected by intranasal application of mouse-adapted influenza A virus A/Mississippi/1/85 (H3N2). The course of infection of immunized mice monitored by the rate of mice survival, the presence of infectious virus and vRNA in lungs, was compared to the control groups of mice pretreated by FA only. All mice survived (100%) in the group immunized with EHA2 and infected with a sublethal dose of virus (0.2 LD<sub>50</sub>) compared to the control where survival was 90%. Infectious virus and vRNA in lungs of immunized mice were present until the 6th day post-infection while in the control mice they were detected two days longer. In the group of mice infected with a higher dose of virus (1.4 LD<sub>50</sub>), 27% survived, while all mice (100%) immunized before the infection survived. Similarly, after the higher dose of infection there was a two-day earlier clearance of virus and vRNA from mice immunized before the infection than in mice infected without immunization.

From these results, we can conclude that HA2 gp is able to induce immune response reducing the severity of infection and replication of virus in lungs of infected animals.

#### Acknowledgements:

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10-003

#### Human cell mediated immune responses induced by a Vero cell derived, inactivated, wild type whole virus H5N1 influenza (A/Vietnam/1203/2004) vaccine.

**Crowe, Brian;** Schwendinger, M.G.; Brühl, P.; Gerencer, M.; Aichinger, G.; Löw-Baselli, A.; Pöllabauer, E.M.; van der Velden, M.; Berezuk, G.; Kistner, O.; Ehrlich, H.J.; Barrett, P.N.

Baxter Innovations GmbH, Austria

It is well established that influenza specific T-cells play an important role in diminishing the lethal effects of and in speeding up recovery from highly pathogenic influenza strains. Thus, particularly for vaccines against highly lethal pandemic H5N1 strains, it might be beneficial if not only protective antibody levels but also influenza specific T cell responses could be induced. To evaluate the cell mediated responses induced by Baxter's Vero cell derived, wild type whole virus H5N1 Influenza (A/Vietnam/1203/2004) candidate vaccine, an exploratory study was performed as part of an open label Phase III study designed to assess the immunogenicity and safety of this vaccine and to investigate the need for and timing of a booster vaccination in adult (18-59 years of age) and elderly (≥60 years of age) subjects. The 40 subjects included in the CMI study were immunized on

day 0 and 21 with a 7.5 µg dose of non-adjuvanted vaccine and boosted on day 180 with either a 3.75 µg or a 7.5 µg dose of non-adjuvanted homologous A/Vietnam/1203/2004 (Clade 1) candidate vaccine or with the same doses of a non-adjuvanted heterologous A/Indonesia/05/2005 (Clade 2.1) candidate vaccine. In the CMI study presented, the T-cell responses, including those from both CD4 and CD8 cells, as well as the plasma cell and the memory B cell responses were monitored for all subjects over a 6-month period. Cryopreserved PBMC preparations obtained on days 0, 10, 28, 180 and 187 were used to monitor vaccine-specific and heterologous cross-clade T cells' responses by an interferon-gamma (INF-γ) Elispot assay, while a flow cytometry based intracellular INF-γ assay discriminated CD4 and CD8 cells. Numbers of vaccine specific and recombinant H5 haemagglutinin (rH5 A/Vietnam/1203/2004) specific plasma cells and memory B cells were determined on days 10, 28 and 187 and on days 0 and 180, respectively, using an IgG Elispot assay. Results of the T-cell INF-γ Elispot analysis clearly showed that the primary immunizations induced considerable numbers of H5N1 Influenza A/Vietnam/1203/2004 specific INF-γ secreting T-cells on day 10 and 28. While the numbers of vaccine specific T-cells did drop off slightly 5 months after the second immunization, they were still maintained at levels significantly higher than before vaccination. The booster immunization at day 180 led to increased numbers of vaccine-specific T-cells after 7 days. A similar, though slightly lower, INF-γ T-cell response against the heterologous A/Indonesia/05/2005 was observed, indicating that a strong cross-clade cross reacting T-cell response was induced by the A/Vietnam/1203/2004 vaccine. Comparable homologous and heterologous T-cell responses were also observed for both the 18-59 and >60 age groups suggesting that inactivated whole virus vaccine induced equivalent T-cell responses in the adult and in the elderly populations. The results of the INF-γ CD4 intracellular cytokine analysis agreed closely with the Elispot results and revealed that the majority of the vaccine specific T-cell responses observed in the Elispot analysis arose from CD4 helper cells. In contrast to the extensive CD4 responses, no significant increase in numbers of vaccine specific CD8 cells could be detected. While the PBMC preparations obtained at days 0 and 180 did not contain detectable numbers of H5N1-specific plasma cells, considerable numbers were detected on day 10 and 28, i.e. 10 and 7 days after the first and second immunizations, respectively. On day 187, 7 days after the booster immunization, the number of H5N1 specific (both homologous and heterologous vaccine specific) and rH5 haemagglutinin (A/Vietnam/1204/2004) specific plasma cells were greater than that seen on day 28, showing the presence of a true secondary booster response to the whole virus and to the H5 haemagglutinin antigen, the primary target for neutralizing antibodies. A comparison of the number of H5N1 vaccine specific memory B cells on day 0 and 180 indicated that the initial 2 shot immunization scheme used in the trial induced a significant and long-lasting increase in the frequencies of both H5N1 viral antigen specific and H5 haemagglutinin specific memory B cells. In conclusion, the Vero cell derived, inactivated,

wild type whole virus H5N1 Influenza (A/Vietnam/1203/2004) vaccine has been shown to induce substantial vaccine specific and cross-clade cross reactive CD4 helper T-cell responses. These responses were also maintained at significant levels 6 months after the first immunization. The frequencies of vaccine-specific CD8 T-cells were not significantly increased. Shortly after each immunization, substantial numbers of H5N1 and rH5 specific plasma cells were observed, the highest numbers being found 7 days after the 6-month booster immunizations. The vaccine was shown to significantly increase the number of H5N1 viral antigen specific and rH5 specific memory B cells 6 months after the first immunization. These responses were also cross-clade cross reactive. Finally, all the CD4, plasma cell and memory B cell responses were seen in the elderly age group (>60 years of age) to the same extent as in the adult age group (18-59 years of age) indicating that the Vero cell derived, inactivated, wild type whole virus H5N1 vaccine is very effective in this potentially at risk population group.

10-004

### Influenza A viruses and the significance of carbohydrates

**Marth, E.<sup>1</sup>; Kleinhapfl, B.<sup>1</sup>; Grisold, A.<sup>1</sup>; Crowe, B.A.<sup>2</sup>; Kistner, O.<sup>2</sup>**

<sup>1</sup>Institute of Hygiene, Microbiology and Environmental Sciences, Austria; <sup>2</sup>Baxter AG, Biomedical Research Center, Austria

**Introduction:** Influenza A viruses (IAV) express two envelope proteins, hemagglutinin (HA) and neuraminidase (NA), which are responsible for the virulence of the IAV. The HA of the IAV is a homotrimer glycoprotein with an ectodomain composed of a stalk region and a globular head to which oligosaccharides are bound by N-linked glycosylation. Some of these carbohydrates are responsible for the folding of the HA. Since 1968, H3N2 has gradually increased the potential number of N-linked glycosylation sites which has led to an attenuated virus (1).

The presence of carbohydrates at the HA can have either positive or detrimental effects on the virus. For example, if oligosaccharides are positioned close to the cleavage site of the HA0, it interferes with the proteolytic activation of the nascent HA0, which interferes with the formation of HA. Alternatively, replication and release of the IAV may be facilitated by carbohydrates which are located next to the receptor binding site. It has been shown that carbohydrates are especially important for the interaction of HA and NA, requiring a balance between receptor binding activity and virus release. The oligosaccharide change at the globular head of the HA is also connected with the "antigenic drift" of the IAV (1).

The innate immune system with its many proteins plays an important role in fighting the IAV in naïve hosts (2). The most

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important components of the innate immune system are I IFNs, TNF, the collectins and defensins. TNF $\alpha$  is produced by PBMC and also responsible for the development of fever. Collectins are a family of lectins in mammals with a collagen-like region. The surfactant protein D (SP-D) leads on the one hand to an inhibition of adsorption of virus and on the other hand to a limitation of the effects of the inflammation in the initial days of the infection. SP-D plays a special role in antigen presentation through dendritic cells.

### Material and methods:

**Material:** Formol-inactivated IAV (H5N1, H3N2, H1N1, subunits) provided by Baxter Vaccines (3).

**Whole blood stimulation [ex vivo test]:** The whole blood of 2 male and 2 female test people was stimulated with the different inactivated influenza viruses or subunits of IAV. For whole blood stimulation, inactivated IAVs were added in a final concentration of 4.5  $\mu$ g/ml calculated on the HA amount. The suspension was added to 250  $\mu$ l RPMI 1640 media supplemented with 100U/ml penicillin and 100g/ml streptomycin, 2mM glutamine and 10% heat inactivated fetal calf serum (FCS, all from Gibco, Paisley, UK) and 250  $\mu$ l heparinized blood and incubated at 37°C in a humidified 95% air / 5% CO<sub>2</sub> atmosphere under permanent horizontal shaking for different times. Following incubation, the samples were centrifuged for 10 min at 1000 x g. The concentration of TNF- $\alpha$  were then determined using Luminex® (Bioplex® TNF $\alpha$  Single Plex, Bio-Rad Lab. Inc.) according to prescribed procedure.

**Enzymatic Deglycosylation:** Oligosaccharide are N-(asparagine)-linked to the HA. For the enzymatic hydrolysis, several endoglycosidases were used. Endoglycosidase F1 (N-Degly Endo F1® Sigma St. Louis), preferring oligosaccharide with a high mannose content as is the case in HA, were most suitable. 200  $\mu$ g glycoprotein final concentration calculated on the HA amount were incubated with 2 $\mu$ l Endo F1 bei 37°C for 1 hour. The glycosylated viruses were separated in SDS PAGE and used in the ex vivo test.

**Endotoxin Test:** For all enzymatic products, endotoxin was determined using the LAL test and the fever reaction was measured in the rabbit. All products were negative both in the LAL test and in the rabbit test, so that the TNF- $\alpha$  was solely attributable to the activation of the toll-like receptors by the virus and its products.

**Results:** In the ex vivo test, the inactivated IAV (H5N1, H3N2, H1N1, subunits) showed different amounts of induced TNF $\alpha$ . The induction of TNF $\alpha$  was clearly increased by deglycosylation. While the untreated viruses resulted in a mean induction of 405  $\pm$  169.9 pg TNF $\alpha$  (H5N1) and 1.040  $\pm$  370.6 pg (Vaxigrip®), the deglycosylated products induced a mean of 7.810  $\pm$  2080 pg (H5N1) and 5.404  $\pm$  718pg TNF $\alpha$  (Vaxigrip®). The TNF $\alpha$  concentration curve is that of a non-linear regression and is observed in receptor bindings.

In the PAGE, the whole virus H5N1 showed significant differences in the electrophoretic mobility due to glycosylation. While in the non-glycosylated virus H5N1, M1-protein and the HA1 appeared

in one band, following deglycosylation of the H5N1, the HA1 and the M1 protein appeared as separate bands. Hemagglutinin HA1 of the other products (H3N2, H1N1 and subunits) showed the identical spectrum in the PAGE prior to and after glycosylation.

**Discussion:** The innate immune system which plays an important role in fighting influenza viruses, especially in the initial phase of the infection, is produced by the cells through the activation of different receptors such as toll-like receptors. TNF $\alpha$  is an important product of this reaction with PAMPs, which is produced as a result of the reaction of different toll-like receptors and the subsequent signal transduction (4). One significant criterium which is always described in new influenza viruses (pandemic viruses) is the observation of the “cytokine storms”. In the case of H3N2, whose carbohydrates have been thoroughly investigated over decades, it has been shown that the virus has become an attenuated virus over the years as a result of the increase in the degree of glycosylation. The development of virus mutations has provided impressive proof of this process (1). We have been able to show that the enzymatic cleavage of carbohydrates from the virus leads to a dramatic increase – of up to 20 times!!! – in the induction of TNF $\alpha$ . This situation is reminiscent of the “cytokine storm” of the IAV H1N1 in 1918 and of H3N2 in 1968.

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10-005



### The role of neutrophils in acute lung injury during H5N1 influenza virus infection

**O'Brien, K.B.<sup>1</sup>**; Carlson, C.M.<sup>2</sup>; Jones, J.C.<sup>2</sup>; Schultz-Cherry, S.L.<sup>2</sup>

<sup>1</sup>University of Wisconsin-Madison, USA; <sup>2</sup>University of Wisconsin, USA

Little is known about the innate immune response to influenza viruses; especially to the highly pathogenic avian influenza H5N1 viruses in mammals. As compared to commonly circulating influenza virus, infection with highly pathogenic avian H5N1 viruses typically leads to severe lung pathology and progressive viral pneumonia. This is commonly associated with significant alterations in circulating macrophages and neutrophils and higher circulating pro-inflammatory cytokine levels. Further, ex vivo and in vitro experiments demonstrated aberrant inflammatory responses to H5N1 viral particles as compared to H1N1 or H3N2 viruses resulting in increased pro-inflammatory molecule production and inflammation. The goal of our studies was to compare the kinetics of the non-intrinsic innate immune response in mice infected with H1N1 or H5N1 influenza viruses. Our preliminary analyses demonstrated increased numbers of neutrophils circulating early in highly pathogenic H5N1 influenza viral infections as compared to H1N1 infections. This was accompanied by changes in the levels of neutrophils in the lungs depending on the viral strain tested. Most surprisingly, all of the highly pathogenic H5N1 viruses induced higher levels of neutrophil-specific activity within the lungs as compared to H1N1 viral infections. The mechanism behind the difference in neutrophils activity is currently under investigation but appears to be related to transforming growth factor-beta activation. Our goal is to gain further information on the innate immune response to H5N1 influenza and its role in acute lung injury during influenza infection.

10-007

### Haemagglutinin cleavage site mutants as live vaccines against influenza A and B viruses

**Stech, J.<sup>1</sup>**; Garn, H.<sup>2</sup>; Herwig, A.<sup>2</sup>; Gabriel, G.<sup>3</sup>; Dauber, B.<sup>4</sup>; Wolff, T.<sup>4</sup>; Mettenleiter, T.C.<sup>1</sup>; Klenk, H.D.<sup>2</sup>

<sup>1</sup>Friedrich-Loeffler-Institut, Germany; <sup>2</sup>Philipps-Universität Marburg, Germany; <sup>3</sup>University of Oxford, UK; <sup>4</sup>Robert-Koch-Institut, Germany

Beside the pandemic potential of HPAIV, both influenza A and B viruses give rise to yearly epidemics in humans accompanied with high mortality and morbidity. As an alternative approach for live vaccines against influenza A and B viruses, we generated by reverse genetics elastase-dependent haemagglutinin cleavage site mutants from the influenza A laboratory strain A/WSN/33 (H1N1), from the high-pathogenic mouse-adapted influenza A strain SC35M (H7N7) and from the influenza B strain B/Lee/40. These mutants were strictly elastase-dependent, grew in cell culture equally well as their corresponding wild-types, and were attenuated in mice. After one intranasal immunization at 10<sup>6</sup> pfu dosage, the mice survived the lethal challenge with wild-type virus without weight loss or other signs of disease; no challenge virus could be detected in mouse lungs. Vaccination with homosubtypic or heterosubtypic influenza A reassortants led to cross-protection. These observations demonstrate that a mutated haemagglutinin dependent on elastase cleavage can serve as an attenuating component of a live vaccine against influenza A or B viruses.

10-008

### Natural infection of humans with clade 2.2 H5N1 influenza viruses induces cross-clade neutralizing antibodies in human sera

**Lin, Y.P.<sup>1</sup>**; Gregory, V.<sup>1</sup>; Yilmaz, N.<sup>2</sup>; Nasidi, A.<sup>3</sup>; Harry, T.<sup>3</sup>; Olalaye, D.<sup>4</sup>; Daniels, R.<sup>1</sup>; Hay, A.<sup>1</sup>

<sup>1</sup>MRC National Institute for Medical Research, UK; <sup>2</sup>Department of Virology, Refik Saydam Hygiene Institute, Ankara, Turkey; <sup>3</sup>National Clinical Research Training Centre, Federal Ministry of Health, Abuja, Nigeria; <sup>4</sup>Department of Virology, University of Ibadan, Nigeria

Natural infection of humans with clade 2.2 H5N1 influenza viruses induces cross-clade neutralizing antibodies in human sera. Yipu Lin, Victoria Gregory, Rod Daniels, Alan Hay. Virology Division, MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK. The avian H5N1 influenza subtype

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encompasses a diverse array of viruses that can be subdivided into numerous antigenically and genetically distinct clades and subclades. A number of these have shown zoonotic potential and to date viruses of clades 1, 2 and 9 have been confirmed in cases of H5N1 infection of humans. For sera derived from such cases, limited information is available regarding the presence of antibodies with cross-clade/subclade neutralization activity. Human sera obtained from individuals with confirmed and unconfirmed cases of H5N1 infection (clade 2.2) were screened for the presence of neutralizing antibody against a variety of candidate H5N1 vaccine strains representative of clade 1 (A/Vietnam/1194/04), clade 2.1 (A/Indonesia/5/05), clade 2.2 (A/bar-headed goose/Qinghai Lake/1A/05 and A/turkey/Turkey/1/05), clade 2.3.4 (A/Anhui/1/05) and clade 9 (A/Hong Kong/213/03). Analyses were performed using ELISA or plaque reduction based micro-neutralization assays and haemagglutination inhibition assay (HI). These assays gave comparable results but the plaque reduction assay proved more sensitive in comparison with the ELISA based micro-neutralization assay and HI. Overall, results showed that natural infection of humans with clade 2.2 H5N1 viruses induced neutralizing antibodies acting against a range of clade 2.2 viruses, a proportion of which were able to neutralize viruses representative of other H5N1 clades and subclades.

10-009

### *In vitro* modelling of human influenza infection

**Hoeve, Marieke<sup>1</sup>**; Franz, S.<sup>1</sup>; Wickert, S.<sup>1</sup>; Nash, A.A.<sup>2</sup>; Dransfield, I.<sup>1</sup>

<sup>1</sup>University of Edinburgh, Centre for Inflammation Research, UK; <sup>2</sup>University of Edinburgh, Centre for Infectious Disease, UK

Influenza virus infection induces the development and progression of inflammatory responses in the lung. In particular, the production and release of pro-inflammatory cytokines like IL-1, IL-6 and TNF- $\alpha$  from inflammatory cells contribute to the cytokine 'storm' associated with infection. These cytokines affect important innate immune effector functions, with key consequences for host defence. Here, we have analysed immediate cellular responses following influenza virus infection to gain insight into innate immunity to influenza.

We developed *in vitro* culture models for virus infection of human epithelial and inflammatory (lung) cells, including pro- and anti-inflammatory macrophages. Using our models we analysed (i) the cellular sources of inflammatory cytokines and chemokines, (ii) the mechanisms of their induction, and (iii) the functional consequences of their production. Cellular responses that impact on anti-viral host immunity have been determined by analysis of histology/morphology (microscopy), mRNA (real time RT-PCR),

protein production (Western Blot), surface marker expression (flow cytometry; microscopy) and production/secretion of pro- and anti-inflammatory cytokines, including IL-1, IL-6, IL-10, IL-12 and TNF $\alpha$  (ELISA of culture supernatants). As viral infection could directly affect macrophage differentiation, phagocytic function and apoptotic programmes, we have also assessed the mechanisms involved in the recognition and subsequent phagocytosis of influenza-infected (apoptotic) epithelial cells by various macrophage populations (using flow cytometry and microscopy).

We will present data on the potential for influenza to regulate cytokines and inflammatory cell effector functions. Our data contribute towards understanding the initiation and progression of inflammation following influenza virus infection in humans and form a foundation for interfering with the development of pathology following human influenza virus infection.



10-010

### Cytotoxic T lymphocytes in heterosubtypic immunity against influenza A viruses

**Bodewes, Rogier<sup>1</sup>**; Kreijtz, J.H.C.<sup>2</sup>; de Mutsert, G.<sup>2</sup>; Fouchier, R.A.M.<sup>2</sup>; Osterhaus, A.D.M.<sup>2</sup>; Rimmelzwaan, G.F.<sup>2</sup>

<sup>1</sup>Department of Virology, Netherlands; <sup>2</sup>Department of Virology, ErasmusMC, Rotterdam, Netherlands

Cytotoxic T lymphocytes (CTLs) play a major role in the control of viral infections, including those caused by influenza viruses. In humans and in mice, it has been shown that influenza virus-specific CTLs were able to reduce virus titers in the lung and morbidity upon infection. In the light of the pandemic threat, there is a major interest in heterosubtypic immunity against different influenza strains. We evaluated the role of CTLs in an influenza mouse-model. First, we infected mouse with influenza A virus X-31 (H<sub>3</sub>N<sub>2</sub>) and subsequently we challenged them with a lethal dose of influenza virus A/PR/8/34 (H<sub>1</sub>N<sub>1</sub>). These viruses contain the same internal proteins, but different surface glycoproteins. Mice primed with influenza A X-31 cleared the infection with influenza A/PR/8/34 in an accelerated fashion, developed less clinical signs and displayed a reduction of lesions in the lungs resulting in improved survival rates of these mice compared to the naïve mice. The improved outcome of the challenge infection correlated with priming for anamnestic virus-specific CD8<sup>+</sup> CTL responses as was demonstrated by the detection of CTLs specific for the H-2Db restricted NP366-374 epitope that was shared by the influenza viruses X-31 and A/PR/8/34. Essentially the same results were obtained when mice were primed with the human influenza virus A/HK/2/68 (H<sub>3</sub>N<sub>2</sub>) and subsequently challenged

with a highly pathogenic avian influenza virus of the H5N1 subtype, this combination better reflected the situation in the human population. Indeed human CTLs directed against human influenza viruses cross-reacted with epitopes derived from H5N1 influenza viruses and were able to recognize and kill target cells infected with H5N1 viruses. Thus, previous exposure to epidemic influenza strains might induce specific CTL responses, which may provide at least partial protection against heterosubtypic influenza A virus strains.

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Kreijtz *et al.*, Vaccine, 2006.

Kreijtz *et al.*, Journal of Virology, in press.

10-011

### Humoral response to influenza neuraminidase in patients with coronary artery disease - a substudy of the FLUCAD study

**Brydak, Lidia B.<sup>1</sup>**; Romanowska, M.<sup>2</sup>; Ciszewski, A.<sup>3</sup>; Bilinska, Z.T.<sup>3</sup>; Nowak, I.<sup>2</sup>; Ruzyllo, W.<sup>3</sup>

<sup>1</sup>National Influenza Center, National Institute of Public Health - National Institute of Hygiene, Poland; <sup>2</sup>National Influenza Center, National Institute of Public Health - National Institute of Hygiene, Warsaw, Poland; <sup>3</sup>Institute of Cardiology, Warsaw, Poland

In 2004-2005, a study on influenza vaccination in secondary prevention from coronary artery disease (FLUCAD study) was carried out. This was a single center, randomized, prospective, double blind, placebo controlled study performed in a group of 658 patients with coronary artery disease (mean age 59.9) (CAD). The randomization schedule was 1:1 (placebo: influenza vaccine). In autumn 2004, 325 patients received subunit influenza vaccine ('Influvac', Solvay Pharma.), and 333 patients received placebo. In the previous report of a FLUCAD study, the authors presented the clinical results showing that influenza vaccination is an independent factor reducing the incidence of coronary ischemic events in CAD patients. In another report, humoral response to influenza hemagglutinin was also presented. The aim of this substudy was to assess antibody response to influenza neuraminidase in serum samples obtained from the blood samples collected before administration of the vaccine or placebo and 8 to 10 weeks after this intervention. Antineuraminidase (anti-NA) antibody levels were measured in sera by a neuraminidase inhibition test performed according to Aymard-Henry's method modified by Douglas A.R. with fetuin used as a substrate for neuraminidase. The following influenza reference strains recommended by the WHO as the vaccine components for the season 2004/05 were used: A/New Caledonia/20/99 (H1N1), A/Christchurch/28/03 (H3N2) and B/Jiangsu/10/03. Serum samples were serially diluted

1:10 up to 1:160. Anti-NA antibody titers were read as the serum dilutions causing 50% inhibition of neuraminidase activity. A neuraminidase inhibition test was done only for those patients from whom paired sera were obtained, i.e. for 78 patients who received vaccine and for 97 patients who received placebo. The following parameters were calculated: geometric mean titer (GMT) of antibodies before and after vaccination and mean fold increase (MFI) of antibody titers after vaccination. For all calculations the results lower than 10 (undetectable antibody levels) were expressed as 5. Statistical analysis was performed by the following statistical tests: W Shapiro-Wilk test, non-parametric Wilcoxon paired test and U Mann-Whitney unpaired test in the STATISTICA data analysis software system (StatSoft, Inc. 2001, version 6.0, USA). Before administration of the vaccine/placebo, GMTs of anti-NA antibodies were similar in both study groups and ranged from 8.3 to 8.9 in the vaccinated patients and from 8.0 to 8.4 in the placebo patients. After vaccination, GMTs were significantly higher than before vaccination and ranged from 31.5 to 36.9, while MFIs of anti-NA antibody levels were between 3.5 and 4.2. After administration of the placebo, GMTs increased when compared with values registered before this intervention and ranged from 11.1 to 11.5, while MFIs amounted to 1.4 for all antigens. After administration of the vaccine/placebo anti-NA antibody levels were significantly higher in the vaccinated group than in the placebo group. There were no differences in the response for different types/subtypes of neuraminidase antigens (N1, N2, NB) with the exception of the vaccinated patients who had pre-vaccination anti-N2 antibody levels significantly higher than anti-NB antibody levels. Influenza vaccine induced the production of anti-NA antibodies in patients with coronary artery disease, which together with anti-HA antibodies provided these patients with protection against influenza infection and post-influenza complications.

10-012



### Dendritic cells are necessary for iBALT formation following influenza virus infection

**Geurts van Kessel, Corine<sup>1</sup>**; Willart, M.A.M.<sup>2</sup>; Muskens, F.<sup>3</sup>; Osterhaus, A.D.M.<sup>4</sup>; Rimmelzwaan, G.F.<sup>4</sup>; Lambrecht, B.N.<sup>2</sup>

<sup>1</sup>Dept. of Pulmonary Med. / Dept. of Virology, Netherlands; <sup>2</sup>Department of Respiratory Diseases, University Hospital Ghent, Belgium; <sup>3</sup>Dept. of Pulmonary Med., Netherlands; <sup>4</sup>Dept. of Virology, Netherlands

Bronchus-associated lymphoid tissue (BALT) was originally described as a mucosal lymphoid organ in the lungs of some species. However, while the lungs of naive mice typically lack BALT, pulmonary influenza infection in mice leads to the development of inducible BALT (iBALT), which is located in peribronchial, perivascular, and interstitial areas throughout the lung.

We have studied the kinetics of iBALT development in the lung following influenza virus infection and found the presence of organized structures containing T cells, B cells and dendritic cells (DCs). Within these lesions we found evidence for follicular DCs and NP-specific B cells, suggesting local class switching. Next, we investigated the role of DCs in iBALT structures by using a CD11c-DTR transgenic mouse model. Depletion of CD11c+ DCs from the airways led to a loss of organized iBALT structures in the lung, and reduced hemagglutinin-specific serum antibodies. The main function of DCs however, was not to present an antigen to T cells but to produce homeostatic chemokines involved in iBALT induction.

All together these results give a clear description of iBALT formation in the lung of influenza-infected mice and show the crucial role of DCs in maintenance of these structures.

10-013



### Identification of HLA class I ligands during influenza A H1N1 and H3N2 infection

**Wahl, Angela<sup>1</sup>**; Schafer, F.<sup>1</sup>; Bardet, W.<sup>1</sup>; Buchli, R.<sup>2</sup>; Eckerd, A.<sup>1</sup>; Air, G.<sup>1</sup>; Hildebrand, W.H.<sup>1</sup>

<sup>1</sup>University of Oklahoma Health Sciences Center, USA; <sup>2</sup>Pure Protein, LLC, USA

In the absence of an effective influenza neutralizing antibody response, cytotoxic T-lymphocytes (CTL) recognize and kill infected cells upon recognition of viral peptide-Human Leukocyte Antigen (HLA) class I complexes displayed on the cell surface. While neutralizing antibodies are directed against the highly variable surface glycoproteins hemagglutinin and neuraminidase, CTL are primarily directed against peptides derived from more conserved internal viral proteins. Identification of influenza peptide-HLA complexes that mark the surface of infected cells has important vaccine and therapeutic implications. To date, only a handful of influenza class I peptides have been identified by reverse immunologic methods. To identify class I ligands that mark infected cells, we collect soluble HLA-B\*0702 molecules from influenza infected and uninfected cells. Peptide ligands and their class I carriers are size separated, uninfected and infected peptide pools are fractionated by RP-HPLC, and peptides are comparatively mapped via mass spectrometry. Infection with two influenza A H1N1 strains, PR/8/34 and Oklahoma/7485/00-01, and one influenza A H3N2 strain, Oklahoma/309/05, yielded identification of seven influenza HLA-B\*0702 ligands derived from nucleoprotein, matrix protein, and hemagglutinin molecules. While infection with all strains resulted in presentation of the previously described B\*0702 nucleoprotein 418-426 ligand, the presentation of four viral B\*0702 ligands is strain dependent and correlates with the level of viral gene expression during infection. Currently, we are testing the T-cell immunogenicity of all directly discovered influenza B\*0702 ligands by ELISPOT in a cohort of HLA matched individuals naturally infected with the circulating H1N1 or H3N2 influenza virus during the 2007-2008 influenza season.



## Innate and adaptive immune response to influenza in ferrets

**Pillet, S.<sup>1</sup>;** Meunier, I.<sup>1</sup>; Somo-Youmbi, É.<sup>1</sup>; Obojes, K.<sup>1</sup>; Kobinger, G.<sup>2</sup>; Gray, M.<sup>2</sup>; von Messling, V.<sup>1</sup>

<sup>1</sup>INRS - Institut Armand-Frappier, Canada; <sup>2</sup>Special Pathogens Program, National Microbiology Laboratory, Public Health Agency of Canada, Canada

**Background:** Seasonal influenza A causes an acute respiratory infection with high morbidity and considerable mortality, mostly in the very young and elderly. The innate immune response is in large part responsible for virus control and clearance, as antibodies and specific T cells are first detected around four days after infection, when the virus titer is already decreasing. It is characterized by a cascade of pro-inflammatory cytokines including type I interferons (IFN), tumor necrosis factor alpha (TNF $\alpha$ ) and interleukins (IL)-6 and -8. Human volunteer studies have shown a direct correlation between disease severity, the extent of viral replication in the upper respiratory tract, and the levels of these cytokines in nasal wash fluids and plasma. In fact, a dysregulated cytokine response is thought to be a decisive factor in recent H5N1 fatalities (de Jong *et al.*, 2006).

Aside from non-human primates, ferrets are considered one of the best influenza animal models because they are naturally susceptible to human strains, and the course and signs of disease reproduce those seen in human patients (Herlocher *et al.*, 2001). However, the lack of suitable immunological reagents has limited the use of this model for the assessment of the host response. To address this issue, we adapted real-time RT-PCR assays for cytokine mRNA quantification to nasal wash cells and developed a variety of immunological tests to assess cellular and humoral immune responses.

### Objectives:

- Identify immune correlates of influenza disease severity.
- Characterize the ferret immune response to influenza.

**Materials and Methods:** Groups of six, four to six month old, male ferrets, seronegative for circulating influenza strains, were used for all experiments. Animals were infected intranasally with 10<sup>5</sup>TCID<sub>50</sub> of one of the following human influenza isolates: H1N1 A/USSR/90/77 (USSR/77), H1N1 A/Puerto Rico/8/34 (PR/34), H3N2 A/Port Chalmers/1/73 (PC/73) and H3N2 A/Aichi/2/68 (Aichi/68). Nasal washes were collected every day for the first four days and every second day thereafter. Virus titers in these fluids were determined by limited dilution, and the remaining sample was used for RNA isolation. The relative induction of IFN $\alpha$ , IFN $\gamma$ , TNF $\alpha$ , IL-6 and IL-8 mRNAs was subsequently quantified by real-time RT-PCR (Svitek and von Messling, 2007).

To characterize the adaptative immune response in more detail, a group of six ferrets was infected with USSR/77 and blood samples were collected weekly for two months post-infection. A small

aliquot of blood was fixed in PBS with 1% paraformaldehyde for a peripheral blood leukocyte count using a no-lyse, no-wash flow cytometric method. The antiviral cellular immune response in peripheral blood mononuclear cells (PBMC) was evaluated by IFN $\gamma$  ELISPOT assay using peptide pools derived from the haemagglutinin (HA), neuraminidase (NA) or nucleo- (NP) proteins of a H1N1 subtype. To ensure that the IFN $\gamma$  induction reflects an activation of the specific cell-mediated immune response, the relative intracellular levels of IFN $\gamma$  in peripheral blood T cells was assessed by FACS analysis. The local and systemic humoral immune response was quantified by an Ig subtype-specific ELISA and hemagglutination inhibition assay.

**Results:** While PR/34 and Aichi/68 had no effect on the body weight and caused only short-lived mild respiratory disease, USSR/77 and PC/73 were associated with a transient weight loss, depression and frequent sneezing, coughing and mucous to purulent nose exudate for at least three days. With the exception of Aichi/68, which replicated more than tenfold less efficiently in the upper respiratory tract, all viruses reached similar nasal wash titers during the first two days after infection. However, the more virulent strains USSR/77 and PC/73 resulted in higher titers at the later infection stages and delayed clearance. Histopathologic analysis of different lung regions revealed a more severe swelling of the alveolar membranes, infiltration of mixed inflammatory cells, and occasional edema in animals infected with the more virulent strains. The cytokine mRNA quantification in nasal wash cells indicated that strains causing a mild disease were associated with a rapid upregulation of IFN $\alpha$ , IFN $\gamma$  and TNF $\alpha$  concomitant with a strong increase of IL-8, while severe infections were characterized by a lower induction of type I and II IFNs but a strong IL-6 upregulation. Influenza infection in ferrets also reproduces the early transient depletion of peripheral blood lymphocytes observed in humans. Virus-specific serum IgM and nasal IgA were detected as early as three days after infection, followed by a strong sustained IgG response beginning at day seven. IFN $\gamma$  production upon in vitro exposure to peptides covering the NA, HA, and NP proteins was first observed seven days post-infection and reached its maximum value after ten days. Although several peptides derived from each of the three proteins were able to promote IFN $\gamma$  synthesis, the NP protein elicited the strongest response. FACS analysis of similarly stimulated PBMCs demonstrated that CD3-positive cells were the main IFN $\gamma$  producers.

**Conclusions:** Our results support the essential role of the innate immune response in the control of the virus spread and disease severity. The observed similarities between our findings and published case reports and human volunteer studies demonstrate the value of the ferret model not only for virulence assessment but also for the evaluation of the host response. The newly developed tools will be an invaluable asset in the ongoing effort to develop novel vaccines and antivirals.

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10-015

### IgG-binding Fc receptors and alveolar macrophages are involved in anti-M2e antibody-mediated immune protection

**Saelens, Xavier<sup>1</sup>**; El Bakkouri, K.<sup>1</sup>; Descamps, F.<sup>1</sup>; De Filette, M.<sup>1</sup>; Smet, A.<sup>1</sup>; Verbeek, S.<sup>2</sup>; Fiers, W.<sup>1</sup>

<sup>1</sup>UGent and VIB, Belgium; <sup>2</sup>Department of Human Genetics, Leiden University Medical Center, Leiden, Netherlands

Matrix protein 2 (M2) is a type III membrane protein with an extracellular domain (M2e) of 23 amino acid residues that is strongly conserved across influenza A virus strains. M2e has gained much interest for vaccine development because it can induce heterosubtypic immunity. Phase I clinical M2e vaccine trials have recently been initiated. Although it is known that M2e-specific IgGs are essential for protection by M2e-based vaccines, its mechanism of action is largely unknown. Here we show that mouse serum antibodies raised by vaccination with an M2e-virus like particle construct specifically bind to influenza virus infected cells as well as to cells that were transduced with an M2-expression lentiviral construct. Anti-M2e antibodies failed to provide protection in FcRgamma knock out animals, indicating that an antibody-dependent cell-mediated effector mechanism is responsible for protection in vivo. By using CD16 and CD64 single and double knockout mice, we demonstrate that both these activating Fc receptors are involved in anti-M2e-mediated immune protection. This result is in agreement with a passive transfer experiment using fractionated anti-M2e ascites in wild type mice, which indicated that anti-M2e IgG1 and IgG2a could protect naive mice from a lethal influenza A virus challenge. Finally, by specifically depleting alveolar macrophages using chlorodanate-loaded liposomes from mice passively immunised with total anti-M2e ascites or sera, we demonstrate an essential in vivo role for these cells. We conclude that Fc receptor-mediated effector mechanisms involving at least alveolar macrophages are important for anti-M2e mediated immune protection in the mouse model.

10-016

### The superior immunogenicity of whole inactivated virus influenza vaccines is mediated by innate immune reactions driven primarily by triggering of Toll-like receptor 7

**Huckriede, Anke<sup>1</sup>**; Geeraedts, F.C.G.<sup>1</sup>; Goutagny, N.<sup>2</sup>; Hornung, V.<sup>2</sup>; Severa, M.<sup>2</sup>; de Haan, A.<sup>1</sup>; Pool, J.<sup>1</sup>; Wilschut, J.<sup>1</sup>; Fitzgerald, K.A.<sup>2</sup>

<sup>1</sup>Department of Medical Microbiology, Molecular Virology Section, University Medical Center Groningen, Netherlands; <sup>2</sup>Department of Medicine, Division of Infectious Diseases and Immunology, University of Massachusetts Medical School, Worcester, USA

For the rational design of new influenza vaccines, it is imperative to unravel vaccine parameters which determine the magnitude and the quality of the vaccine-induced immune response. In clinical trials, whole inactivated virus (WIV) vaccines were found to induce higher serum hemagglutination inhibition (HI) titers and virus neutralization titers in unprimed individuals than split virus (SV) and subunit (SU) vaccine. Similarly, WIV is more immunogenic than SV and SU vaccine in mice and induces a Th1 type of immune response characterized by low IgG1, high IgG2a/2c and a high number of IFN $\gamma$ -producing T-cells. This phenotype of the immune response is similar to the one induced during infection and is considered as optimal for protection and rapid virus clearance. In contrast, immunization with SV and SU vaccine raises a typical Th2 response characterized by high IgG1, low IgG2a/2c, and a low number of IFN $\gamma$ -producing T-cells. We set out to unravel the parameters responsible for the superior immunogenicity of WIV focusing on the presence in WIV of single stranded (ss) viral RNA, a well-known pathogen-associated molecular pattern (PAMP) of viruses.

In order to investigate the role of the ssRNA, wild-type (wt) mice and mice with a knock-out mutation for either Toll-like receptor 7 (TLR7<sup>-/-</sup>) or a mutation for the TLR adaptor proteins MyD88 and TRIF (MyD88/TRIF<sup>-/-</sup>) were immunized with WIV, SV, or SU vaccine prepared from the H5N1 vaccine strain NIBRG-14. TLR7 is a pattern recognition receptor present in endosomes of (plasmacytoid) dendritic cells (pDCs) and B cells which specifically recognizes ssRNA. When immunized with WIV TLR7<sup>-/-</sup> mice and MyD88/TRIF<sup>-/-</sup> mice developed similar but significantly lower serum HI titers and H5N1-specific IgG levels than WIV-immunized wt mice. Furthermore, the amounts of H5N1-specific IgG2c and the numbers of Th1 cells were strongly decreased in WIV-immunized TLR-ko mice. In contrast, immunization with either SP or SU vaccine resulted in identical antibody titers and immune phenotypes in TLR7<sup>-/-</sup>, MyD88/TRIF<sup>-/-</sup> and wt mice indicating that TLR-dependent mechanisms do not play a role in immune reactions to these vaccines. These data demonstrate that ssRNA is the key component in WIV which triggers the observed heavy production of antibodies and the Th1-skewing of the immune response by engagement of TLR7. However, even in TLR-ko mice WIV remained more immunogenic than split and subunit vaccines

indicating the involvement of additional TLR-independent mechanisms. In vitro experiments with pDCs showed that IFN $\alpha$ , a cytokine involved in Th1-skewing and IgG2a/2c isotype switching, was induced only by WIV but not by the other vaccines. WIV-induced production of IFN $\alpha$  was found in pDCs purified from wt but also from TLR7 $^{-/-}$  splenocytes. TLR7-independent production of IFN $\alpha$  is probably triggered by cytosolic recognition of ssRNA via RIG-I and might contribute to the immunogenic potential of WIV in wt as well as in TLR-ko mice.

In conclusion, the superior immunogenicity of WIV as compared to SV and SU vaccines is most likely caused by the viral ssRNA present in WIV but not in the other vaccines. The viral ssRNA, a well-known PAMP, activates the immune system predominantly via TLR7-dependent but also via TLR-independent mechanisms. Our study provides evidence for direct links between innate and adaptive immune responses to influenza vaccines and proves an important role for TLRs in governing responses to the long-established WIV vaccine formulation. These insights can be of eminent importance for the development of improved dose-sparing influenza vaccine formulations urgently needed in case of a new pandemic.

## 11 CLINICAL & EPIDEMIOLOGICAL VACCINE EVALUATION

11-001

### Randomized trial comparing immunogenicity and safety of reduced dose intradermal administration of 2007/2008-season influenza vaccine with full dose intramuscular administration in young adults\*

**Kuenzi, V.<sup>1</sup>; Kompier, R.<sup>2</sup>; Kuersteiner, O.<sup>1</sup>; Seiberling, M.<sup>3</sup>; Weverling, G.J.<sup>2</sup>; Goudsmit, J.<sup>2</sup>**

<sup>1</sup>Crucell, Berna Biotech Ltd, Berne, Switzerland; <sup>2</sup>Crucell, Leiden, Netherlands;

<sup>3</sup>Swiss Pharma Contract Ltd, Allschwil, Switzerland

**Background:** Antigen-presenting cells such as dendritic cells and macrophages play a role in initiating an effective immune response, and are represented at higher concentrations in the skin than in the muscle. Intradermal (i.d.) administration of influenza vaccine could therefore potentially elicit a more robust antibody response than standard intramuscular (i.m.) administration, hence lower antigen doses may be required with i.d. vaccination, for comparable efficacy.

**Objectives:** To evaluate the immunogenicity and safety of three reduced doses of 2007/2008-season virosomal adjuvanted influenza vaccine administered i.d. compared with full dose i.m. administration (Inflexal® V) in healthy young adults, in a Phase II, single-center, randomized trial. The present analysis is for a subset of the main trial.

**Methods:** 224 healthy female and male adults aged  $\geq 18$  to  $\leq 60$  years (mean 38.4 years) were allocated to four groups. On Day 1, subjects received single doses of influenza vaccine (2007/2008-season) as follows: Groups 1, 2 and 3 were given 0.1 mL influenza vaccine containing reduced doses of 3.0, 4.5 and 6.0  $\mu$ g hemagglutinin antigen (HA) of each influenza virus strain, respectively, via i.d. injection (hypodermic needle); Group 4 received 0.5 mL of Inflexal® V i.m. at full dose (15  $\mu$ g HA of each strain). Serum anti-influenza virus antibodies were determined by a hemagglutination inhibition assay on Day 1 and Day 22  $\pm$  2 days. Safety was assessed for 3 weeks after vaccination (including a 4-day adverse event questionnaire). The humoral immune parameters and safety were evaluated according to the criteria described in the EMEA guideline "Note for Guidance on Harmonisation of Requirements for Influenza Vaccines", 1997.

**Results:** The EMEA requirements set for assessment of influenza vaccines in adults aged 18 to 60 years were fulfilled in all groups, irrespective of dose and mode of administration. Very high seroconversion rates were observed in all four study groups: 51-86% in Group 1, 68-83% in Group 2, 60-84% in Group 3, and 70-87% in Group 4. In addition, marked increases in geometric mean titer (GMT) were observed across the groups three weeks after vaccination for all influenza virus strains, with GMT fold-increases

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of 38, 70, 47 and 56 for Groups 1 through 4, respectively, for the A/Solomon Islands strain. Both i.d. and i.m. administration were well tolerated; reported adverse reactions were generally considered to be mild to moderate and resolved within a few days. Systemic reactions were broadly similar across the groups. With respect to local reactions, injection site pain was more prevalent with i.m. than i.d. vaccine administration; other local reactions were more frequent with i.d. than i.m. vaccine administration, as expected due to the mechanism of action for the i.d. administration route.

**Conclusion:** Virosomal adjuvanted influenza vaccine was highly immunogenic and well tolerated when given i.d. at reduced doses, eliciting an immune response similar to that observed with full dose i.m. administration. Reduced dosing of virosomal adjuvanted influenza vaccine thus suggests a promising antigen-sparing strategy for universal influenza vaccination against endemic influenza, and would help to meet the global need for increased amounts of antigen in the event of a pandemic.

\*Preliminary data, prior to database lock

11-002

### Interventions to improve influenza vaccination coverage among children with chronic asthma

**Esposito, Susanna<sup>1</sup>**; Gasparini, C.<sup>1</sup>; Pelucchi, C.<sup>2</sup>; Bellasio, M.<sup>3</sup>; Tel, F.<sup>3</sup>; Semino, M.<sup>3</sup>; Sabatini, C.<sup>3</sup>; Chiarelli, G.<sup>3</sup>; Principi, N.<sup>3</sup>

<sup>1</sup>Institute of Pediatrics, University of Milan, Fondazione IRCCS "Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena", Italy; <sup>2</sup>Mario Negri Institute, Milan, Italy;

<sup>3</sup>Institute of Pediatrics, University of Milan, Fondazione IRCCS "Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena", Italy

**Background and aims:** Although health authorities recommend influenza vaccination in patients with chronic asthma, the level of vaccination coverage in these patients remains very low.

**Methods:** This study involved patients with chronic asthma whose parents were administered a questionnaire eliciting information regarding influenza vaccination status of their children. Thereafter, all the children were randomized in three different groups: group A, directly called by a doctor who is not the same one they usually refer to and vaccination given by external operators; group B, called directly by the doctor they usually refer to, but vaccination given by external operators; group C, called and also vaccinated directly by doctors working in the outpatient clinic for chronic asthma.

**Results:** Among 348 children with chronic asthma (225 males; mean age, 10.2 ± 3.4 years), 141 children (40.5%) had received the vaccine at least once in their life and 125 (35.9%) repeated it in the season before the interventions. All the three different interventions increased influenza vaccination coverage, although

there was a variation in this increase of 11-21%. Group C reached the highest vaccination coverage value regardless of asthma severity, children's age and previous influenza vaccination status ( $p=0.005$ ; OR 2.0<sup>3</sup>; 95% CI, 1.02-4.05).

**Conclusions:** Without specific interventions, the rate of delivery of influenza vaccine to children with chronic asthma appears inadequate. In order to improve influenza vaccination coverage, specific interventions appear mandatory in these high risk patients. The administration of influenza vaccine during routine clinical visits performed in asthma follow-up appears the best way to increase influenza vaccination coverage.

11-003

### An influenza B outbreak in elderly vaccinated people in the winter season 2007/08

**Camilloni, B.<sup>1</sup>**; Neri, M.<sup>1</sup>; Lepri, E.<sup>1</sup>; Sigismondi, N.<sup>2</sup>; Iorio, A.M.<sup>1</sup>

<sup>1</sup>Department of Surgery Specialities and Public Health, University of Perugia, Italy; <sup>2</sup>Nursing Home Bartolomei Castori, Foligno, Italy

**Aim:** The aim of the study was to investigate the antibody immune response induced by a trivalent influenza vaccine and its ability to prevent influenza infections in the 2007/08 winter season, characterised by a prevalent co-circulation of influenza A/H1N1 and B viruses distinguishable from the corresponding vaccine components.

**Methods and results:** Sixty-seven female elderly (mean age 84 years, range 61-102 years) volunteers living in a nursing home in Umbria, a region of central Italy, received one dose of commercially available trivalent influenza MF59-adjuvanted vaccine (FLUAD, Novartis) in November 2007. Each dose of vaccine contained 15 microg of A/Wisconsin/67/05 (H3N2), A/Solomon/3/06 (H1N1) and B/Malaysia/2506/04.

Vaccine immunogenicity was evaluated measuring haemagglutination inhibiting antibody (HI) titres in sera collected before (day 0) and one month (day 30) after immunisation. The vaccine induced statistically significant increases in the numbers of seroprotected people (HI titres  $\geq 1:40$ ) and in the values of geometric mean titres (GMT) against all three vaccine antigens. The post-vaccination requirements of the European Commission for acceptability of influenza vaccination in elderly subjects were always satisfied. After vaccination, the percentages of seroprotected volunteers ranged between 61.2 and 85.1%, the mean fold increase of GMT between 2.7 and 4.8 and the percentages of people showing a positive response between 41.8 and 56.7%.

In the first week of March 2008, the occurrence of influenza-like illness (ILI) was reported in 11 vaccinated people. A new blood sample (day 120) was collected from these volunteers and

the HI titre was compared with results found in sera collected respectively before and one month after vaccination against the three vaccine antigens. Comparing HI titres found in day 120 and day 30 sera, no seroconversions were observed against A/H3N2 and A/H1N1 vaccine antigens. Five people showed seroconversion against influenza B virus vaccine antigen. Four of the five people were volunteers who had not reached protective antibody titres one month after vaccination.

Throat swabs from 6 of the 11 volunteers with ILI were collected and examined for the presence of influenza viruses, and influenza B virus circulation in the nursing home was laboratory confirmed.

**Discussion:** Although the immunogenicity of the 2007/08 influenza vaccine was acceptable according to the criteria of the European Commission, consistent percentages of people did not reach protective antibody titres after vaccination. The highest values of unprotected people were found against A/H1 (34.3%) and B (38.8%) vaccine antigens. This situation allowed the circulation of influenza B viruses, probably antigenically drifted (the results of the antigenic and genetic characterization are not still available) as compared with vaccine antigen, in the vaccinated volunteers of the nursing home examined.

11-004

#### Role of various factors of immunity in protection against influenza after vaccination of Live Attenuated Influenza Vaccine (LAIV)

**Rudenko, Larisa;** Naikhin, A.N.; Donina, S.A.; Chirkova, T.V.; Petukhova, G.D.; Desheva, Y.A.; Rekstin, A.R.

*Institute of Experimental Medicine RAMS, Russian Federation*

Until now, the major criterion of influenza vaccine efficacy has been the results received in epidemiological trials in large groups of humans. (Ph III clinical trials).

These epidemiological trials are conducted on the strength of safety and immunogenicity results from trials on limited groups of volunteers (Ph. I/II). Immunogenicity criteria are the percentage of seroconversions, GMT after vaccination as well as percentage of people with a protective titre of antibodies  $\geq 1:40$ , based on hemagglutination inhibition tests in serum.

New highly pathogenic influenza viruses, types H5, H7 and H9, have appeared, and these are potential candidates for a future pandemic. By their very nature, it is impossible to evaluate them in epidemiological trials. This has led to a requirement to broaden the research to cover correlation of protection.

The research includes studies of the vaccines' spectrum of activity against drifted variants of influenza viruses; improvement of the existing, and development of new, evaluative methods for

immunogenicity; and understanding the role of various factors of immunity in protection against infection.

This work will present data on correlation between anti-hemagglutination and secretory antibodies in protecting against influenza infection, and the influence of functional activity of such antibodies on influenza illness after vaccination with LAIV and with inactivated vaccine. One of the major components of effective vaccines in general and influenza vaccines in particular is memory cell induction.

The present study will analyse memory/effector T-cells' stimulation of immune responses to live attenuated reassortant influenza vaccine on volunteers. It has been shown that intranasal immunization of young adults with LAIV leads to increases in the amount of the peripheral blood CD4 and CD8 + T-cells with CD 45 RO+ phenotype.

BioDiam Ltd. of Melbourne, Australia, provided financial support for this study.

11-005

#### Safety, tolerability and immunogenicity of two trivalent subunit inactivated influenza vaccines: a phase III, observer-blind, randomized, controlled multicenter study

**Fragapane, E.<sup>1</sup>;** Groth, N.<sup>2</sup>; Hilbert Anke, H.A.<sup>3</sup>; Casula, D.<sup>2</sup>; Tregnani, M.<sup>4</sup>

<sup>1</sup>Influenza Vaccine Development, Novartis Vaccines & Diagnostics, Italy; <sup>2</sup>Novartis Vaccines & Diagnostics S.r.l., Siena, Italy; <sup>3</sup>Novartis Vaccines & Diagnostics GmbH & Co. KG, Germany; <sup>4</sup>Centro De Desarrollo De Proyectos Avanzados, Rome, Italy

**Background and Aim:** The most effective method for reducing the number of influenza cases each season is annual vaccination and the Centers for Disease Control (CDC) and Prevention's Advisory Committee on Immunization Practices (ACIP) now recommends influenza vaccination for people 50 years of age or older, healthy children aged 6 months to 18 years of age, and those in close contact with children aged 0-59 months. Increased demand for influenza vaccines in the United States has resulted in vaccine supply shortages in past influenza seasons. This demand could be met by influenza vaccines licensed in other jurisdictions which already have a large post-marketing database. Agrippal™ is a subunit, inactivated, egg-based trivalent influenza vaccine authorized in Italy in 1986. The authorization was extended to 15 European Union countries in 1998, and today it holds marketing authorizations in more than 50 countries worldwide. Since 1997, nearly 73 million doses have been sold resulting in an excellent pharmacovigilance and post-marketing profile. This phase 3 study, carried out in healthy participants from 3 to 64 years of age, evaluated the safety, clinical tolerability, and immunogenicity of Agrippal™ compared with Fluvirin (already

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in use in the United States) according to the FDA/CBER Guidance for Industry issued in May 2007.

**Methods:** In this observer-blind, randomized, controlled, multicenter study, carried out between April and December 2007 in Argentina, participants were randomized (2:1) to received either Agrippal (n=1262) or Fluvirin (n=631) stratified according to age. Adolescents (9 to 17 years) and adults (18 to 64 years) received one dose and children (3 to 8 years, un-primed) received 2 doses 4 weeks apart. Each vaccine contained 15 µg of viral hemagglutinin (HA) for each of the virus strains recommended for the 2007 Southern Hemisphere influenza season: A/New Caledonia/20/99 (H1N1)-like, A/Wisconsin/67/2005 (H3N2)-like, and B/Malaysia/2506/2004-like. Hemagglutinin inhibition (HI) titres were measured pre-vaccination and 21 days post vaccination, for adolescents and adults, or 28 days post first dose and 21 days post second dose for children. Pre- and post-vaccination geometric mean antibody titres (GMTs), seroprotection rates (percentage of subjects achieving a hemagglutination inhibition [HI] titer  $\geq 40$ ), seroconversion rates (percentage of subjects achieving seroconversion defined as negative pre-vaccination serum [HI<10]/ post-vaccination HI titer  $\geq 40$ ; or significant increase defined as at least a 4-fold increase in titer from non-negative pre-vaccination serum [HI $\geq 10$ ]), were calculated and assessed according to FDA/CBER criteria (FDA/CBER Guidance for Industry issued in May 2007).

**Results:** Baseline characteristics and demographics were evenly distributed between the two vaccine groups in each age stratum. After one injection, at least one solicited local or systemic reaction was reported in approximately half of adolescents (42% Agrippal, 41% Fluvirin) and adults (50% Agrippal, 55% Fluvirin) and in approximately one third of children (32% Agrippal, 37% Fluvirin). Most reactions were mild to moderate. Baseline seroprotection rates against the two vaccine strains of influenza A ranged from 54 to 74% in children, from 60 to 80% in adolescents and from 35 to 41% in adults. Baseline seroprotection rates against the influenza B strain were lower, ranging from 15% to 20% across the three age groups. In adolescents and adults, a single dose of Agrippal or Fluvirin elicited a response to all three vaccine strains that met the CBER criteria for seroprotection (lower limit of the two-sided 95% CI  $\geq 70\%$ ) and for seroconversion (lower limit of the two-sided 95% CI  $\geq 40\%$ ). In children, a single dose of either vaccine was sufficient to meet CBER criteria for the influenza A strains, while a second dose was required to achieve sufficient levels of seroprotection against the influenza B strain.

**Conclusion:** In this large Phase III clinical study, Agrippal demonstrated a safety and tolerability profile comparable to that of Fluvirin. Both vaccines also met the FDA/CBER criteria for immunogenicity. The purity of subunit vaccines explains their favorable tolerability profile, and supports the use of Agrippal and Fluvirin to vaccinate the general population on an annual basis, including with those at high risk, such as children and the elderly.

11-006

### Influenza vaccine delivery programs: a systematic review

Sander, B.<sup>1</sup>; Mir, M.<sup>2</sup>; Krahn, M.<sup>1</sup>

<sup>1</sup>University of Toronto, Canada; <sup>2</sup>University Health Network, Canada

**Background:** Annual epidemics of influenza continue to cause worldwide morbidity, mortality and societal disruption. Jurisdictions utilize a mix of influenza delivery strategies to reach vaccine coverage targets with varying success.

**Objective:** This systematic review examines influenza vaccine immunization delivery programs and settings around the world to identify and describe successful programs.

**Methods:** We searched MEDLINE and EMBASE databases to identify records describing the methods of delivering influenza vaccine to the population at large. A total of 1844 records were initially screened and 98 citations were selected as potentially relevant and obtained in full-text. Detailed information was abstracted using a pre-specified 20-item data extraction form.

**Results:** Most influenza immunization programs are targeted to vaccinate those at high risk of complications from influenza infection, as well as their contacts in traditional settings such as physician offices. However, an increasing number of people are receiving the influenza vaccine in non-traditional settings such as pharmacies, schools and in the workplace. Immunization programs in non-traditional settings are often more accessible and convenient, especially for the economically disadvantaged, inner city, and minority populations.

**Conclusion:** Health care providers' offices, hospitals and public health units continue to effectively deliver influenza vaccines to the general population.



11-007

### Efficacy of trivalent influenza vaccine during the 2007-08 season of A & B mismatch

Taylor, Marsha<sup>1</sup>; Skowronski, D.M.<sup>2</sup>; Petric, M.<sup>2</sup>; Dickinson, J.<sup>3</sup>; Fonseca, K.<sup>4</sup>; De Serres, G.<sup>5</sup>; Charest, H.<sup>5</sup>; Drews, S.J.<sup>6</sup>; Crowcroft, N.<sup>6</sup>; Winter, A.<sup>7</sup>; Bontovics, E.<sup>7</sup>; Kwindt, T.L.<sup>2</sup>; Bastien, N.<sup>8</sup>; Li, Y.<sup>8</sup>

<sup>1</sup>Canadian Field Epidemiology Program, Public Health Agency of Canada, Canada;

<sup>2</sup>BC Centre for Disease Control, Canada; <sup>3</sup>University of Calgary, Canada; <sup>4</sup>Alberta

Provincial Laboratory, Canada; <sup>5</sup>Quebec National Institute of Public Health,

Canada; <sup>6</sup>Ontario Public Health Laboratory, Canada; <sup>7</sup>Ontario Ministry of Health

and Long-Term Care, Canada; <sup>8</sup>National Microbiology Lab of Canada, Canada

**Introduction:** Trivalent inactivated influenza vaccine (TIV) is reformulated annually to include representative seed strains of two influenza A subtypes (H3N2 and H1N1) and one of two influenza B lineages (Victoria or Yamagata). During the 2007-08 season in the Northern Hemisphere, a notable mismatch of the A/H3N2 and B components to circulating viruses was reported. We measured efficacy of the 2007-08 TIV using a case-control approach applied to linked sentinel physician networks in four Canadian provinces.

**Methods:** TIV for the 2007-08 season in Canada included A/Solomon Islands/3/2006(H1N1)-like, A/Wisconsin/67/2005(H3N2)-like and B/Malaysia/2506/2004-like (Victoria lineage) components. Participants were patients  $\geq 9$  years of age who presented with influenza-like illness (ILI) to a sentinel physician in British Columbia, Alberta, Ontario or Quebec, Canada between November 2007 and April 2008. Cases were participants in whom influenza was identified by PCR or culture. Controls presented with ILI but tested negative for influenza A and B. Isolates were characterized by gene-sequencing and hemagglutination-inhibition (HI) assays. Odds ratios (OR) for influenza in vaccinated versus non-vaccinated persons were derived with appropriate adjustments. VE was estimated as 1-OR.

**Results:** By March 18, 2008, 862 participants had been recruited to the study: 49% from BC, 36% from Alberta, 7% from Ontario and 8% from Quebec. Median age of participants was 34 years, 7% were  $\geq 65$  years and 19% received the 2007-08 vaccine at least two weeks prior to ILI onset. 392 (45%) were positive for influenza; 66% typed as influenza A. Influenza A/H1N1 (well-matched to vaccine) and influenza B/Yamagata (lineage mismatch) predominated with a late-season increase in H3N2 (strain mismatch to vaccine). Study recruitment continued until April 30, 2008. Subtype-specific vaccine efficacy estimates with adjustment will be presented and compared to findings from the previous season.

**Conclusion:** With regional representation and broad-based sentinel contribution, trivalent vaccine efficacy estimates can be derived annually and correlated with a match to circulating influenza variants. Since sentinel networks exist in most countries of both hemispheres, participation could be expanded to better inform vaccine selection and the monitoring of vaccine performance globally.

## Local antibody immune response to live attenuated reassortant influenza vaccine: generation of secretory IgA and their avidity

Donina, S.A.; Korenkov, D.A.; **Chirkova, T.V.**; Naykhin, A.N.; Rudenko, L.G.

*Institute of Experimental Medicine RAMS, Russian Federation*

**Background:** The effectiveness of antibody immune response depends on antibody titers as well as their functional activity (avidity). Avidity defines the reaction rate of antibody with antigens and the strength of antibody-antigen bonding. Immunogenicity of influenza vaccine is currently evaluated by measuring serum anti-haemagglutination antibody levels. However, local immunity, particularly sIgA antibodies, plays the main role in protection against respiratory infection. It has been shown that one of the advantages of live attenuated reassortant influenza vaccine (LAIV) is the stimulation of local immunity in the upper respiratory tract. This study provides a comparative analysis of the sIgA generation rate and avidity of these antibodies in young adults immunized with LAIV.

**Materials and methods:** Young adults aged 18–22 were immunized with LAIV or received placebo (physiological solution). Nasal secretions were collected from volunteers before and 21 days after immunization. Geometric mean titers (GMT) of virus-specific sIgA were measured by the ELISA method. Secretory IgA avidity was evaluated using two methods: (i) kinetic ELISA test, based on dynamic measurement of sIgA titers subject to timing of serum contact with virus antigen; (ii) ELISA with chaotropic agent (carbamide) which reveals the strength of antibody-antigen bonding. Both ELISA tests evaluating sIgA were specific to A(H1N1) virus included in LAIV.

**Results:** Virus-specific sIgA GMTs in the LAIV group were 32.8% and 79.4% before and after vaccination respectively, and 58.6% of vaccinated volunteers had a measurable increase of IgA titers ( $\geq 4$  fold). In the placebo group, there was no any increase of IgA GMT. Avidity of sIgA by the kinetic ELISA test was assessed by dynamic measurement of antibody titers after 0.25, 0.5 and 1 hour of contact of serum with the virus antigen. The dynamic avidity index (dAI) defines the antibody-antigen reaction rate. It ranged from 1 to 13 units. A significant increase of sIgA avidity (in 2.5 – 8.0 times) after immunization with LAIV was shown, particularly in volunteers with low dAI before vaccination. 65.5% of volunteers had a high dAI (8 – 13), 17.2% had a low dAI (1 – 3) and 17.2% had a medium dAI (4 – 7). In the placebo group, volunteers with high, medium and low dAI were found in equal measure (up to 35%). Another characteristic of antibody-antigen reaction is the strength of bond formation. This was investigated in an assay with a chaotropic agent. The avidity index in this

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method (cAI) is the percent of retained antibody-antigen bonds after carbamide exposure, and cAI also varied widely (45 – 95 units). Volunteers with medium cAI (61 – 80) were found in vaccine and placebo groups in equal measure (up to 65%), while there were significantly more volunteers with a high cAI (81 – 95) in the LAIV group (27.6%) than in the placebo group (9.9%). To the contrary, a low cAI (45 – 60) predominated in the placebo group (31%) compared to 6.9% in the LAIV group. As in dynamic avidity testing, this study showed a significant increase of cAI (up on 16 – 31%) in the LAIV compared to placebo administration. We also found that only the dAI directly correlated with sIgA GMTs, in contrast to the cAI whose value was not dependant on antibody amount.

**Conclusion:** LAIV significantly stimulated the increase of quantitative and qualitative characteristics of local antibody immune response in young adults – levels of both sIgA and avidity. More than 50% of vaccinated volunteers showed an increase in sIgA titers concurrent with the elevation of avidity. BioDiam Ltd., of Melbourne, Australia, provided financial support for this study.

11-010

### FLUSECURE: pandemic influenza vaccines for the European population

**Schmidt, Ed<sup>1</sup>**; Brooks, T.<sup>2</sup>; Wolff, T.<sup>3</sup>; Socan, M.<sup>4</sup>; Visontai, I.<sup>5</sup>; Onu, A.<sup>6</sup>; Oftung, F.<sup>7</sup>; Kilpi, T.<sup>8</sup>; Agger, E.<sup>9</sup>; van der Werf, S.<sup>10</sup>; Soethout, E.<sup>11</sup>; van der Zeijst, B.<sup>11</sup>

<sup>1</sup>Netherlands Vaccine Institute NVI, Netherlands; <sup>2</sup>Health Protection Agency, UK; <sup>3</sup>Robert Koch Institute, Germany; <sup>4</sup>Slovenia National Institute of Public Health, Slovenia; <sup>5</sup>National Center for Epidemiology, Hungary; <sup>6</sup>Cantacuzino Institute, Romania; <sup>7</sup>Norwegian Institute of Public Health, Norway; <sup>8</sup>National Institute of Public Health, Finland; <sup>9</sup>Statens Serum Institute, Denmark; <sup>10</sup>Institut Pasteur, France; <sup>11</sup>Netherlands Vaccine Institute, Netherlands

FLUSECURE represents an EU-funded network of 10 European health institutes. This consortium provides new tools, technologies and services for pandemic influenza vaccine development with a focus on shortening the lead time necessary for production: This is achieved by the following actions:

1. Establishment of an extensive library of pandemic vaccine reference strains against pandemic influenza threats
2. Production of the corresponding reagents, necessary for production by industry
3. Increasing the yield of pandemic vaccines during production
4. Comparative testing of all available influenza adjuvants
5. Development of promising new adjuvants
6. Development and validation of improved correlates of protection

7. Establishment of a trial network of clinical study centers throughout Europe
8. Trial studies on pandemic influenza vaccines

The products and services of this EU consortium are made available to both governments and industries. FLUSECURE offers a complete pipeline for pandemic vaccine development and testing.

At this stage the consortium has already generated a series of new pandemic vaccines and reagents based on three different H5N1, H7N1, H7N3, H9N3 and H9N2. An H2N2 vaccine is currently being produced. A large scale comparative evaluation of influenza adjuvants has been completed. A number of new serological and cellular assays for vaccine efficacy testing has been developed already and is being validated. For 2008, several pandemic trial studies have been scheduled already.

11-011

### Safe administration of an inactivated virosomal adjuvanted influenza vaccine in asthmatic children with egg allergy

**Esposito, Susanna<sup>1</sup>**; Gasparini, C.<sup>2</sup>; Martelli, A.<sup>3</sup>; Zenga, A.<sup>3</sup>; Tremolati, E.<sup>4</sup>; Varin, E.<sup>4</sup>; Marseglia, G.<sup>5</sup>; Principi, N.<sup>4</sup>

<sup>1</sup>Institute of Pediatrics, University of Milan, Fondazione IRCCS "Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena", Italy; <sup>2</sup>Institute of Pediatrics, University of Milan, Fondazione IRCCS Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena", Italy; <sup>3</sup>Pediatric Unit, Ospedale Macedonio Melloni, Milan, Italy; <sup>4</sup>Institute of Pediatrics, University of Milan, Fondazione IRCCS Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena, Italy; <sup>5</sup>Department of Pediatrics, University of Pavia, IRCCS Policlinico San Matteo, Italy

**Background and aims:** Administration of influenza vaccination in asthmatic children with egg allergy is largely debated for the fear of severe allergic adverse events (AEs). Recently marketed vaccines contain small amounts of egg proteins but it is not known whether they could be used in children with egg allergy without the risk of AEs.

**Methods:** A total of 44 children with chronic asthma and egg allergy and 44 children with chronic asthma and no egg allergy were enrolled. The severity of egg allergy was defined on clinical data and egg-specific tests (14 patients had mild allergy, 19 moderate and 11 severe). All the children performed skin testing with the influenza vaccine, they received a whole intramuscular dose of a virosomal influenza vaccine (Inflexal V, Berna Biotech) and were monitored for AEs in the 28 days after vaccination.

**Results:** Skin testing with influenza vaccine was always negative and prevalence of AEs was similar among children with and without egg allergy, regardless of its severity. Mild, transient immediate AEs were detected in two children with egg allergy (4.5%) and one without (2.3%), local late AEs in 7 with egg

allergy (15.9%) and 6 without (13.6%), systemic late AEs in 19 with egg allergy (43.2%) and 18 without (40.9%) and asthma recurrences in 4 with egg allergy (9.1%) and 7 without (15.9%). No serious AE was reported.

**Conclusion:** Children with chronic asthma and egg allergy can safely receive a whole dose of virosomal adjuvanted influenza vaccine in a one-dose protocol without any significant risk of AE. This appears important for increasing vaccination coverage of asthmatic patients and in case of influenza pandemic.

11-012

### Universal influenza immunization program coverage rates in Ontario children

**Moran, K.<sup>1</sup>; Maaten, S.<sup>2</sup>; Kwong, J.<sup>2</sup>; Guttman, A.<sup>2</sup>; Northrup, D.<sup>3</sup>**

<sup>1</sup>Durham Region Health Department, ON, Canada; <sup>2</sup>Institute for Clinical Evaluative Sciences, ON, Canada; <sup>3</sup>Institute for Social Research, York University, ON, Canada

**Background:** Ontario is the only province in Canada with a universal influenza immunization program, offering free vaccination to all residents aged 6 months or older. Coverage rates for Ontario children have never been assessed. The objective of this study was to estimate influenza immunization coverage rates in Ontario children aged 12 years or younger for the 2006-07 influenza season and to compare Ontario rates with those in other provinces that have targeted programs, offering free vaccination only to identified high-risk groups.

**Methods:** From April to September 2007, a household telephone survey was conducted. The person most responsible for caring for the children provided responses to the question, "Since September 2006, has [child] received a flu shot?" Follow up questions determined compliance with the recommended dosage schedule, prevalence of chronic conditions, and other characteristics of the children and respondent. Complete and partial coverage were defined according to Canadian guidelines for influenza vaccination of children. Information was collected on all children aged 6 months to 11 years in the household. High-risk groups included those aged 6 to 23 months and children of any age with chronic medical conditions. Survey estimates for children aged 6 to 23 months were compared with vaccination rates derived from physician billing claims from health administrative data for all Ontarians and also with vaccination rates achieved in other provinces.

**Results:** The study sample included 5,063 children from 3,029 households. The coverage rate (complete and partial combined) was highest in children aged 2 to 11 years with chronic conditions at 34.7% (preliminary un-weighted estimates) (Table 1). The coverage rate in healthy children aged 2 to 11 years was 30.9%. Children aged 6 to 23 months had the lowest coverage rate at

23.2%. The rate of vaccination for children aged 6 to 23 months based on physician billing claims was 11.0%. The Ontario estimate for children aged 6 to 23 months was lower than those obtained in other provinces.

**Table 1: Influenza Immunization Coverage in Ontario Children**

Risk Group	% Complete	% Partial*	% None	Total (n)
6-23 months	10.3	12.9	76.9	800
2-11 years with chronic conditions	30.6	4.1	65.2	728
2-11 years without chronic conditions	24.6	6.3	69.1	3,326
Total	23.1	7.1	69.8	4,854**

\* received only 1 dose when 2 doses were indicated for the child according to Canadian guidelines.

\*\* excludes child proxies born after September 30, 2006 (n=83), missing age (n=4) and 'don't know' responses to "Since September 2006, has [child] received a flu shot?" (n=122).

**Discussion:** These are the first provincial estimates of influenza immunization coverage in Ontario children since the introduction of the universal program. Rates in children in high-risk groups are below the provincial target of 70%. Parent-reported coverage rates for children aged 6 to 23 months are higher than those from administrative data. This may be attributed to parental over-reporting of influenza vaccine received in physician offices, physician under-billing for influenza vaccine given or an under-estimation using physician billing claims as children may be immunized in locations other than physician offices, such as public health clinics and pharmacies. Under Ontario's universal program, preliminary results show that a higher coverage rate in children aged 6 to 23 months has not been realized when compared to estimates from provinces that specifically target this risk group.

11-013

### Efficacy of inactivated virosomal adjuvanted influenza vaccine in preventing Acute Otitis Media in children with a history of complicated or uncomplicated recurrent Acute Otitis Media

**Marchisio, P.; Fusi, M.; Picchi, R.; Dusi, E.; Bianchini, S.; Nazzari, E.; Esposito, S.; Principi, N.**

*Institute of Pediatrics, University of Milan, Fondazione IRCCS Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena, Italy*

**Background and aims:** Influenza vaccine is effective in preventing acute otitis media (AOM) in day-care children and in those with a history of recurrent uncomplicated AOM (rUN-AOM). We evaluated the preventing efficacy of influenza vaccine in children with a history of recurrent AOM complicated by spontaneous

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perforation (rC-AOM).

**Methods:** In November 2006, 180 children aged 1-4 years with a history of rUN-AOM or rC-AOM (at least 3 episodes in 6 months) randomly received an inactivated virosomal adjuvanted influenza vaccine (Inflexal V Berna) or no treatment. During a 6-month follow-up, pneumatic otoscopy and tympanometry were performed every 4-6 weeks and in case of respiratory symptoms.

**Results:** 90 children (48 rUN-AOM, 42 rC-AOM) were vaccinated while 90 (49 rUN-AOM, 41 rC-AOM) were controls. Among 97 children with rUN-AOM, AOM was diagnosed in 20 (41.7%) vaccinated children compared to 39 (79.6%) controls ( $p=0.0002$ ). The mean number of episodes of AOM was  $0.62 \pm 0.87$  in those vaccinated compared to  $2.14 \pm 1.59$  in controls (reduction 70%). Among 83 with rC-AOM, AOM was diagnosed in 29 (69.1%) vaccinated children compared to 35 (85.4%) controls ( $p=0.13$ ). The mean number of episodes of AOM was  $1.31 \pm 1.26$  in those vaccinated and  $2.00 \pm 1.45$  in controls (reduction 34.5%).

**Conclusions:** The preventing efficacy of inactivated virosomal adjuvanted influenza vaccine is significantly greater in children with rUN-AOM compared to those with rC-AOM. In the latter, influenza vaccine does not significantly interrupt the recurrence but it is moderately effective in reducing the number of new episodes.

11-014

### Influenza vaccine coverage in Portugal in 2007/08: lessons learned for the next seasons

**Rebello-de-Andrade, H.<sup>1</sup>; Valente, P.<sup>2</sup>; Nunes, B.<sup>3</sup>; Falcão, J.M.<sup>3</sup>**

<sup>1</sup>Centro Nacional da Gripe, Instituto Nacional de Saúde, Portugal; <sup>2</sup>Direcção Geral da Saúde, on behalf of the Working Group on Influenza, Portugal; <sup>3</sup>Departamento de Epidemiologia, Instituto Nacional de Saúde, Portugal

**Introduction:** Influenza vaccination is an effective way of reducing the mortality and morbidity associated with influenza, especially in the elderly and in patients with high-risk conditions. It may also be associated with significant economic benefits, not only in those groups but also among healthy working adults.

The strategies for implementing vaccination recommendations combine education of health care workers and other potential vaccine recipients, annual information campaigns, efforts in minimising administrative and financial barriers and include systems for monitoring vaccination rates.

The influenza vaccine in Portugal requires a medical prescription and 40% of its cost is supported by the state; in occupational settings the vaccine is paid by the employer.

Aiming at preventing influenza in Portugal, the General Directorate of Health established last winter (2007/2008) a multidisciplinary

working group that includes other institutions from the Ministry of Health, the Pharmaceutical Industry, Pharmacies Association, the Medicine Distributor's Association and the Pharmaceutical Association, to follow and improve the implementation of vaccine recommendations at national level.

**Objectives:** The objectives were to identify the levels of influenza vaccine coverage in Portugal, nationally, by region, age group and risk conditions, and also to evaluate the vaccine coverage in nursing homes (both in residents and staff) and in health professionals from Health Care Centres and Hospitals from the National Health Service.

**Material and Methods:** The influenza vaccine coverage, at the population level, has been obtained from ECOS (1) ("at home we watch health"), a sample of approximately 1000 households surveyed by a computer assisted telephone interview, which was developed by the Departamento de Epidemiologia of the Instituto Nacional de Sa de (INSA). The ECOS sample is a health-region stratified random sample, with homogeneous allocation, where the households have been selected and recruited, respecting the representativeness of the mainland Portuguese families with landline telephone.

The 2007/2008 influenza vaccination survey was carried out from the end of February to the beginning of March 2008. Vaccine coverage estimates were computed by age groups, health region and by the groups of individuals that stated they had been diagnosed by a medical doctor for asthma, diabetes, hypertension and rheumatic disease. All estimates were weighted by health region.

The results presented were obtained with the package of statistical programs *SPSS 15 Base and Complex Samples*.

The General Directorate of Health coordinates the evaluation of the vaccine coverage on both health professionals and Nursing Homes (residents and staff). The evaluation of the vaccine coverage of health professionals, by professional group, is reported by Health Centres and Hospitals at regional and national level at the end of each influenza season.

The evaluation of vaccine coverage of residents and staff of nursing homes was based on an enquiry sent to all Health Centres through the Regional Health Administrations, which provided information on the total number of institutions, the number of institutions that replied, the number of institutionalised patients and staff, and how many of these were vaccinated.

The number of vaccines sold was provided by the Pharmaceutical Industry and the Pharmacies Association.

**Results:** The ECOS estimate of influenza vaccine coverage for mainland Portugal was 16.0% (CI95%: 14.5-17.6) (sample of 2537 individuals). For individuals 65 years of age or older, the vaccine coverage was estimated at 51%. The age group that presented the lowest vaccine coverage (3.9%) was that of individuals younger than 15 years. Individuals that stated they had been diagnosed with *Asthma* and *Diabetes* presented the highest estimates of vaccine coverage, 37.0% and 44.2%, respectively. In terms of geographic distribution, the Northern Health Region presented the higher estimate of vaccine coverage (18.6%).

The influenza vaccine coverage for residents in Nursing Homes was estimated to be 87.6%, while for staff it was only 27.5%.

For health professionals, there was a clear difference in the vaccine coverage in Health Centres and hospitals. In fact, Health Centres revealed 43% of vaccine coverage in Medical Doctors, 51.7% in nursing staff, and 44.4% in other professional categories, whereas for Hospitals the estimates were 27.6%, 36.5% and 36.4%, respectively. Preliminary data indicates that, during the 2007/2008 influenza season, 1.3 million vaccines were sold. Information on vaccine coverage in these specific settings will be updated as soon as final data are available (April).

**Discussion:** The ECOS sample was designed based on the Portuguese population with landline telephone. As such, individuals without telephones or who only possess mobile telephones are not represented in this sample. In the 2007/2008 season, the influenza vaccine coverage in the age group 65+ reached the highest value since 1998/1999. It should be noted that the coverage of this age group has increased consistently from around 30% in 1998 to the current 51% value. However, this is still far from the recommended values already reached by countries such as Spain or the United Kingdom (over 70%) where vaccine is free of charge. In general, coverage of other age groups has been kept stable over the last decade.

Vaccine coverage of diabetic (44.2%) and asthma/COPD (37%) patients was lower than recommended, although the values have also increased consistently over time.

One positive outcome of this work was to demonstrate the high vaccine coverage observed in the residents of nursing homes. On the other hand, the vaccine coverage observed in professional health care workers, as well as in the staff of nursing homes, remains at low levels, a problem that is common to several European countries. Given that these groups are providing care to patients, including those from high risk groups, they are important targets for improving vaccination, not only because this would help preventing transmission of the disease but also because they play an important role in communicating and motivating other population groups to get vaccinated. A major challenge will be to increase vaccination rates in these groups, by exploring factors that may drive vaccination, enhancing the responsibility of the occupational health services within the health care services and nursing homes.

<sup>5</sup> Working Group on Influenza

Campos A, Santos M - Wholesalers Association of Chemical and Pharmaceutical Products (GROQUIFAR); Duarte P - National Association of Pharmacies (ANF); Ferreira M - National Authority of Medicines and Health Products (INFARMED); Freitas G - General-Directorate of Health (DGS); Frutuoso A, Gonçalves E., Santos I, Ferreira H, Cale E, Valente P - 5 Regional Health Administrations (ARS, IP); Lopes C, Lains C - Portuguese Pharmaceutical Industry Association (APIFARMA); Mendonça M - Pharmaceutical Association; Rebelo-de-Andrade H - National Institute of Health (INSA); Rodrigues S - Portugal's Pharmacies Association (AFP)

11-015

### Safety and cross-reactive immunogenicity of two H5N1 A/Indonesia/5/2005 (clade 2.1) AS03-adjuvanted prepandemic candidate influenza vaccines. A phase I/II clinical trial

**Langley, Joanne**<sup>1</sup>; Frenette, L.<sup>2</sup>; Ferguson, L.<sup>3</sup>; Riff, D.<sup>4</sup>; Folkerth, S.<sup>5</sup>; Sheldon, E.<sup>6</sup>; Segall, N.<sup>7</sup>; Risi, G.<sup>8</sup>; Middleton, R.<sup>9</sup>; Johnson, C.<sup>10</sup>; Li, P.<sup>11</sup>; Innis, B.<sup>11</sup>; Fries, L.<sup>11</sup>

<sup>1</sup>Canadian Center for Vaccinology, Canada; <sup>2</sup>Q&T Research, Sherbrooke, Canada;

<sup>3</sup>Colchester Research Group, Truro, Canada; <sup>4</sup>Advanced Clinical Research Institute,

Anaheim, CA, USA; <sup>5</sup>Clinical Research Center of Nevada, Las Vegas, USA; <sup>6</sup>Miami

Research Associates, FL, USA; <sup>7</sup>Clinical Research Atlanta, Stockbridge, GA, USA;

<sup>8</sup>Infectious Disease Specialists, MT, USA; <sup>9</sup>Accelovance, Huntsville, AL, USA;

<sup>10</sup>Johnson County Clinical Trials, Lenexa, KS, USA; <sup>11</sup>Glaxosmithkline, USA

Prepandemic H5N1 vaccines should be antigen-sparing, have acceptable reactogenicity and offer cross-reactive immunity to non-vaccine strains. Most clinical studies have evaluated clade 1 strains which infected humans in 2004-2005, while clade 2 strains have infected humans since 2005. We evaluated two H5N1 vaccine candidates made with a clade 2.1 strain.

The vaccine candidates, produced at 2 sites (Dresden [D], Quebec [Q]) by 2 distinct processes, were made with A/Indonesia/5/2005 (clade 2.1) antigens. Adults (18-64 years) were vaccinated twice, 21 days apart, with 3.75µg of hemagglutinin (HA) with an oil-in-water emulsion-based Adjuvant System (AS03) or without (control). 150 subjects received AS03-adjuvanted D- or Q-antigens, while 75 control subjects received a non-adjuvanted Q-antigen. At days 0, 21 and 42, sera were tested for hemagglutination-inhibiting (HI) antibody against A/Indonesia/5/2005 and A/Vietnam/1194/2004 (Clade 1). Solicited local and general symptoms, unsolicited adverse events (AEs) and serious adverse events (SAEs) were recorded. (110028/NCT00510874).

Although injection site pain was more frequent in the AS03-adjuvanted vaccine groups, redness and swelling occurred in <5% of subjects. General symptoms (day 0-6) were more frequent in the AS vaccine groups but fever was uncommon (≤2%). All AEs decreased after dose 2. Clinical laboratory values and unsolicited AEs raised no safety concerns. No vaccine-related SAEs occurred.

After dose 1, A/Indonesia seroconversion rates (SCR) reached 42.1%-45.7% in the AS vaccine groups (control:6.7%). After dose 2, SCR and the percentage of subjects with HI titers ≥40 were 96.4%-97.2% in the AS03 vaccine groups (control:17.3%). Homologous HI GMT was enhanced ≥44-fold in the pooled AS vaccine group. After 2 doses, SCR to Clade 1 A/Vietnam/1194/04 was 56.4%-62.1% for the AS vaccine groups.

At an antigen-sparing dose, the AS03-adjuvanted A/Indonesia/05/2005 vaccines were markedly immunogenic against the vaccine strain and a Clade 1 strain. Reactogenicity was increased but acceptable, and no other safety concerns were identified.

11-016

# **Influenza vaccination coverage rates in four European countries during the winter of 2007/08**

**Blank, P.R.;** Szucs, T.S.

*Institute of Social and Preventive Medicine, Switzerland*

**Objective:** The objectives of the study were to identify the level of influenza vaccination coverage in four European countries in the season 2007/08, to understand the primary drivers and barriers to vaccination, to assess the major encouraging factors for vaccination as well as vaccination intentions for the next winter 2008/09.

**Methods:** Representative household surveys were conducted with telephone interviews of individuals aged 14 and above. The questionnaire used in the UK, Germany, Italy and Spain was essentially the same. The research was carried out between December 2007 and January 2008.

**Results:** In the season 2007/08, the vaccination rates in the general population remained stable in Germany (28% vs. 28% in 2006), Spain (24% vs. 22% in 2006) and Italy (23% vs. 24% in 2006). The coverage increased by four points in the UK from 25% in 2006/07 to 29% in 2007/08. The proportion of never vaccinated individuals ranged between 46% in Germany and 69% in Italy.

Across all four countries, the most frequent reason for getting vaccinated was the advice from a family doctor or nurse (65%), the perception of flu as a serious illness (56%) or to prevent the transmission to family members or friends (44%). Having forgotten to vaccinate (40%) or not feeling concerned (27%) was the major cause for not getting vaccinated this year among those vaccinated in the season before. Individuals never vaccinated did not think they were likely to catch influenza (46%) or they have never considered it before (39%), whereas concerns of possible side effects from the vaccine were stated by 15%. Across all surveyed countries, the recommendation by the family doctor or nurse (67%) was deemed as the strongest encouraging factor, followed by the reason of travelling to regions with a high risk of influenza (37%) and having more information regarding efficacy (33%).

A total of 35% of the respondents intended to get immunized against influenza in 2008/09 (ranging from 27% in Italy to 43% in Germany).

**Conclusions:** In Germany, Italy and Spain, influenza vaccination coverage rates in the season 2007/08 varied inappreciably compared to the previous influenza season, whereas UK indicated a rate up by 4%. Our survey indicated that most vaccinated individuals were immunized because of the family doctor's recommendation. The reason for non-vaccination was mainly feeling that catching the flu was unlikely. However, the activation of the correct driving forces and dealing with the barriers may help to enhance coverage rates in Europe.

11-017

# **Influenza vaccination reduces the frequency of coronary events in patients with coronary artery disease - FLUCAD study**

**Ciszewski, A.<sup>1</sup>;** Bilinska, Z.T.<sup>1</sup>; Brydak, L.B.<sup>2</sup>; Romanowska, M.<sup>3</sup>

*<sup>1</sup>Institute of Cardiology, Warsaw, Poland; <sup>2</sup>National Influenza Center, NIH, Warsaw. Chair and Dept. of Family Medicine, Medical University of Warsaw, Poland; <sup>3</sup>National Influenza Center, National Institute of Hygiene, Warsaw, Poland*

**Aims:** Influenza vaccination is recommended in patients (pts) with coronary artery disease (CAD), despite a shortage of studies proving its protective effect. We therefore evaluated the effect of influenza vaccination on the incidence of coronary events in pts with angiographically confirmed CAD.

**Methods and Results:** Prospective, randomized, double blind, placebo controlled study. We included 658 optimally treated CAD pts; 477 men, mean age 59.9+/-10.3 years. 325 pts received the influenza vaccine, and 333 pts received placebo. No patient was lost to follow-up. Mean follow-up was 296.8+/-35.7 days. Primary end-point: cardiovascular death occurred in 2 pts (0.61%) in the vaccine vs. in 2 pts (0.60%) in the placebo group (p=NS). Second composite end-point: A Coronary Ischemic Event (MACE or hospitalization for myocardial ischemia) occurred significantly less frequently in the vaccine group: 16 pts (4.9%) vs. 30 pts (9.0%) in controls, OR 0.54 (95% CI, 0.30 to 0.99, p=0.047). By multivariate analysis: recent acute coronary syndrome (HR 2.93, 95% CI 1.52 – 5.65, p=0.0014), influenza vaccination (HR 0.38, 95% CI 0.19 – 0.78, p=0.009), and female sex (HR 2.15, 95% CI 1.11 – 4.15, p=0.0235), were independent predictors of the Coronary Ischemic Event.

**Conclusions:** Influenza vaccination may reduce frequency of Coronary Ischemic Events in pts with coronary disease.

11-018

# **Seroprotection rates in vaccinees correlate with vaccine effectiveness against serologically confirmed influenza infection; Berlin, Germany, 2006/07**

**Buchholz, U.<sup>1</sup>;** Schweiger, B.<sup>1</sup>; Troschke, B.<sup>1</sup>; an der Heiden, M.<sup>1</sup>; Hartwig, M.<sup>1</sup>; Williams, C.<sup>2</sup>

*<sup>1</sup>Robert Koch-Institute, Germany; <sup>2</sup>Norfolk Suffolk and Cambridge Health Protection Unit, UK*

The European Medicines Agency (EMA) uses the proportion of seroprotected vaccinees (seroprotection rate; SPR) as one of three criteria to license seasonal influenza vaccines. The

SPR criterion is satisfied if more than 70% of vaccinees have a titer of 40 or more to the vaccine strain. These criteria have rarely been validated for their predictive value regarding vaccine effectiveness. As part of a study in the season 2006/07 where we compared the risk of serologically confirmed influenza infection (SCII) in healthcare workers and non-healthcare workers, we assessed the correlation of SPR and vaccine effectiveness. During the 2006/07 influenza season we conducted a prospective, multicentre cohort study in people aged 16 to 65 in Berlin, Germany. Recruited participants gave serum samples before and after the season; in vaccinees pre-season serum samples were taken at least 14 days after vaccination, no sample was taken before vaccination. Occurrence of influenza-like illness (ILI), defined as acute illness onset with cough, fever and body pain, was ascertained weekly. As 85% of the viruses circulating during the season 2006/07 belonged to the subtype A/H3N2 and were similar to the vaccine strain A/Wisconsin/67/2005 (H3N2), the haemagglutination inhibition titers against this strain were determined. SCII was defined as a fourfold or greater titer rise between pre- and post-season samples, with a post-season titer of at least 40. Using SCII as outcome and vaccination as exposure we calculated vaccine effectiveness as  $1 - \text{risk ratio}$ . SPR was determined among vaccinees in 6 age groups, and plotted against vaccine effectiveness. Correlation was assessed by the coefficient of determination. 736 individuals were included in the serological analysis. 250 were healthcare workers and 486 non-healthcare workers. Crude vaccine effectiveness was 0.47 ( $p=0.02$ ) for SCII and 0.71 for SCII with ILI. Overall, effectiveness was similar in healthcare workers and non-healthcare workers. SPR by age group showed a decline with linear trend by age with a high of 100% in the 15-19 and 18% in the 60-69 year age group. The group aged 20-29 years had an SPR of 67%. Vaccine effectiveness for A/Wisconsin/67/2005 also showed a linear declining trend with age. Among age groups, SPR and vaccine effectiveness were correlated with a coefficient of determination of 0.83 (Figure). Influenza vaccine was protective against SCII, but offered additional protection against the more severe clinical manifestations of influenza. In this setting, SPR seems to be a valid proxy for vaccine effectiveness.

11-019

### Effect of influenza and pneumococcal vaccines in elderly persons in years of low influenza activity

Sylvan, P.E.S.; Christenson, B.

Department of Communicable Disease Control and Prevention, Uppsala County Council, Sweden

The present prospective study was conducted from 2003-2005, among all individuals 65 years and older in Uppsala County, a region with 300 000 inhabitants located close to the Stockholm urban area. The influenza activity was low in the Uppsala region as in the rest of Sweden during the three influenza seasons while the lowest influenza activity was observed in 2003. The objective of this study was to assess the preventive effect of influenza and pneumococcal vaccination in reducing hospitalisation and length of hospital stay even during periods of low influenza activity. The specificity of the apparent vaccine associations were evaluated in relation to the influenza seasons. In 2003, the total study population was 41,059, of which 12,907 (31%) received influenza vaccine, and of these, 4,447 (11%) were administered the pneumococcal vaccine. In 2004, 14,799 (34%) individuals received the influenza vaccine and 8,843 (21%) the pneumococcal vaccine, while in 2005, 16,926 (39%) individuals were given the influenza vaccine and 12,340 (28%) the pneumococcal vaccine. Our findings indicated that 35% of the vaccinated cohort belonged to a medical risk category and it was mainly those people who received the pneumococcal vaccine. Data on hospitalisation and mortality during the 3-year period were obtained from the Uppsala County council. During the influenza seasons, reduction of hospital admissions and significantly shorter in-hospital stays for influenza were observed in the vaccinated cohort. For individuals who had also received the pneumococcal vaccine, a significant reduction of hospital admissions ( $68\%p<0.005$ ) and of in-hospital stays ( $40\%p<0.001$ ) were observed for invasive pneumococcal disease and of in-hospital stays for pneumococcal pneumonia ( $38\%p<0.001$ ). For cardiac failure, effectiveness was observed even in people who had received the pneumococcal vaccine, despite the fact that the pneumococcal vaccinated belonged to a medical risk category. Reduction in deaths from all causes was observed during the influenza season of 2004, in the 75-84 year old age group and in all age groups during the influenza season 2005. Conclusions: the present study shows that influenza and pneumococcal vaccinations are beneficial for elderly persons, even during periods of low influenza activity. Moreover, the additive effect of the two vaccines in the elderly is associated with reduced risk in hospitalisation and a reduction in the mean length of hospital stays. We also found a significant reduction in mortality during the influenza seasons 2004 and 2005.

11-020

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### Effectiveness of an influenza vaccination implementation program aimed at health care workers in Dutch nursing homes

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**Looijmans -van den Akker, Ingrid<sup>1</sup>**; van Delden, J.J.M.<sup>1</sup>; Hulscher, M.E.J.<sup>2</sup>; van Essen, G.A.<sup>1</sup>; Verheij, T.J.M.<sup>1</sup>; Hak, E.<sup>1</sup>

<sup>1</sup>University Medical Center Utrecht, Netherlands; <sup>2</sup>University Medical Center Nijmegen, Netherlands

**Introduction:** Recent studies have shown substantial benefits from routine influenza vaccination among health care workers (HCWs) in long-term care institutions. Vaccination of these HCWs results in a reduction of influenza morbidity and mortality among patients. In Dutch nursing homes, there has been a guideline for influenza vaccination among HCWs since 2004, but influenza vaccination rates among HCWs remain low in spite of this guideline. Therefore further research to develop an effective influenza vaccination implementation program in order to raise vaccination rates among HCWs is necessary.

**Objective:** Determining the effectiveness of an influenza vaccination implementation program aimed at HCWs in Dutch nursing homes.

**Methods:** In this pragmatic randomised controlled trial, an educational program on influenza vaccination aimed at HCWs was implemented in 16 Dutch nursing homes before the beginning of the influenza season of 2006-2007. This program focused on behavioural and policy determinants which in preceding research have shown to be of significant relevance for acceptance of influenza vaccination among HCWs in Dutch nursing homes. In the control group of 17 nursing homes, the vaccination campaign remained unchanged compared to the previous seasons. After the implementation program the vaccination rate among HCWs in the intervention group was compared to that in the control group.

**Results:** In the intervention group, 25.1% (774 out of 3086) of the HCWs have been vaccinated against influenza after the implementation program compared to 16.4% (582 out of 2968) in the control group. The implementation program has therefore resulted in a significantly higher influenza vaccination rate among HCWs in the intervention group (RR:1.5<sup>3</sup>; 95% CI:1.39-1.68).

**Discussion:** This influenza vaccination implementation program aimed at HCWs in Dutch nursing homes has shown to be effective in raising the vaccination rate among HCWs. The further increase in this vaccination rate is however needed. This can possibly be achieved by adjustments in the implementation program and execution of the program during several consecutive years.

11-022

### Influenza and pneumococcal vaccine coverage in persons aged > 65 years in 2004-2006 in Poland

**Nitsch-Osuch, Aneta;** Wardyn, K.; Gyrczuk, E.

*Department of Family Medicine, Medical University of Warsaw, Poland*

**Introduction:** Persons aged > 65 years should be vaccinated against flu and pneumococcal infections. These vaccinations prevent the diseases and their complications (mostly pneumonia), provide less severe course of the disease, and decrease the mortality and morbidity rates.

**Aim:** The aim of the study was to find the coverage of vaccination against influenza and pneumococcal infections in persons aged > 65 years in Poland in 2004-2006.

**Material and methods:** Data concerning the number of vaccinations against influenza and *Streptococcus pneumoniae* infections, collected in 2004-2006 by the National Institute of Hygiene, National Institute of Public Health and Chief Sanitary Inspectorate, published yearly as a bulletin "Vaccinations in Poland", available on [www.pzh.gov.pl](http://www.pzh.gov.pl), were analyzed. Demographic data were obtained from Central Statistical Office ().

**Results:** Among persons aged > 65 years the influenza vaccine coverage was 7.3; 7.9; 8.6%, respectively in 2004, 2005 and 2006. Persons at this age represented from 25% (in 2005) to 32% (in 2006) of all vaccinated individuals. The percentage of newly vaccinated against pneumococci people aged > 65 years varied from 0.02% (in 2004) to 0.06% (in 2006). The estimated pneumococcal vaccine coverage was 0.1%. Individuals aged >65 years represented from 12.4% (in 2006) to 27.7% (in 2004) of all vaccinated people.

**Conclusions:** The influenza vaccination coverage among persons aged > 65 years in 2004-2006 in Poland was low (mean 8%) while the pneumococcal vaccination coverage was extremely low (0.1%). The influenza and pneumococcal vaccine coverage should be improved in the future.

11-023

### Sensitivity analysis to estimate the potential impact of unmeasured confounding in non-randomized influenza vaccination studies

**Groenwold, R.H.H.<sup>1</sup>;** Nelson, D.B.<sup>2</sup>; Nichol, K.L.<sup>2</sup>; Hoes, A.W.<sup>1</sup>; Hak, E.<sup>1</sup>

*<sup>1</sup>Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht, Netherlands; <sup>2</sup>VA Medical Center, Minneapolis, USA*

Findings from non-randomized studies on the association between rare clinical outcomes and influenza vaccination using large health care databases may be prone to bias resulting from unmeasured confounding. To quantify the potential impact of such bias on the estimated vaccine effects, such as for example the reduction in all-cause mortality by influenza vaccination, statistical simulation techniques are available to perform sensitivity analyses. We developed a generic sensitivity analysis method based on assumed associations between the unmeasured confounder (e.g. functional health status) and exposure status (e.g. influenza vaccination), the unmeasured confounder and outcome (e.g. mortality), and prevalence of the unmeasured confounder in the population (e.g. elderly persons). We applied this method in a non-randomized database study on the association between influenza vaccination and mortality among elderly persons from the Netherlands. Information on patients aged 65 years and older obtained from the computerized Utrecht General Practitioner database over 7 influenza epidemic periods was pooled (n = 44,418 episodes). After adjustment for measured confounders including demographics, co-morbidity, medication use and prior health care use, influenza vaccination reduced mortality by 42% (adjusted odds ratio [OR] 0.58, 95% confidence interval [CI]: 0.46 – 0.73). A high prevalence for the unmeasured confounder should include a high prevalence (40%), and strong independent associations of the confounder with both vaccination status and mortality (OR ≤ 0.3 and OR ≥ 3.0, respectively) are necessary to arrive at an insignificant (P > 0.05) association between influenza vaccination and mortality (OR 0.79, 95%CI: 0.62 – 1.00). We recommend the use of a sensitivity analysis to support statements of the impact of unmeasured confounding bias on reported effects of non-randomized intervention studies.

11-024

# Influenza vaccination: Organized prevention in general practice

Cooman, Kris

General Practitioner, Belgium

**Introduction:** The importance of influenza, as an annually recurring ailment, is large enough to pay attention to the prevention of it. In Belgium, 1500-2000 patients die from the complications of an influenza infection every year. The benefit of vaccination against influenza has been proven for a long time: vaccination can offer up to 70% protection against the worst complications of influenza (such as pneumonia and cardiac failure) and can reduce the mortality by 80%. This means a considerable cost saving for the patient and society.

The aim of our project was to examine if the encoded EMF (Electronic Medical File) with ICPC-codes (International Classification of Primary Care) and the GMF (Global Medical File) were useful instruments for calling up patients and in this way increasing the vaccination level in the target group.

**Method:** In mid-September 2006, lists were made of the patients who would be called. From the total active patient population of 2897 patients, 191 over 65 and 128 patients younger than 65 with risk factors (such as diabetes, heart and lung disease, thyroid gland disease, employment in a child nursery or nursing home, pregnancy in 2° and 3° trimester,...) were selected by using our medical software. Totally 319 patients were selected to call up.

The call up letter informed our patients about the seriousness of an influenza infection and about the benefits of vaccination. An information brochure and a prescription were added. By the end of September the letters were posted. If there was no response by mid-November, a reminder was sent. 109 reminders were sent. Our registration was suspended at the end of December. In January the results were collected and compared with those of another general practitioner who only sent a call up letter (no reminders were sent).

In 2007, we repeated the registration, but this time to all our patients over 50 as well as high risk patients. 541 letters were sent, 480 to our over 50 group and 61 to our risk population. By mid-November, 261 reminders were sent.

**Results:** In 2006, 81% of the patients called up responded. Of these, 74% were vaccinated and 7% refused actively ("have never had the flu" and "became ill after a vaccination" were the most frequent reasons for refusing). 62% of our risk population and 82% of the patients over 65 received a vaccination. The reminder resulted in an increase of 20% in the level of vaccination in the group at risk and 10% in the over 65 group compared to the vaccination level reached by mid-November. On average a 15% increase was achieved as a result of the reminder. The impact of the reminder was the largest in the high risk group.

The response to the project organised by the colleague who

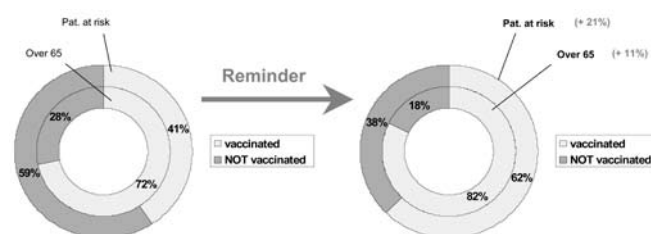
only sent a call up letter was much lower: 58% of the called up patients were vaccinated (41% in the group at risk and 72% in the over 65 group). The response was 21% lower in the group at risk and 10% lower in the over 65 group. We can thus observe the same impact of the reminder. The high risk patients, who need the influenza vaccination the most, respond the least to the call up letter. But, the reminder letter has a larger effect on this group and can result in the desired response.

In 2007, 70.2% of our patients responded, 62.5% were vaccinated (79.6% in the over 65 group, 55.8% in the group aged between 50 and 65, and 32.8% in the high risk group (younger than 50). We can conclude that the younger the patient, the worse the response, even when they are at risk.

**Conclusion:** We have proven several times that a prevention campaign organised within the general practitioners' setting can be an enormous success, in contrast to the prevention campaigns organised by the government. The reason for this is the personalised approach of the general practitioner as the main character within primary care medicine and his/her in-depth knowledge of the patients and their medical history.

A further increase in the vaccination percentage seems difficult to achieve, but in absolute numbers we can still achieve an increase. The absolute number of people vaccinated can only increase when more patients choose to open a GMF. This is only possible by a systematic approach to opening a GMF or by automatic renewal by the health insurance fund.

The ICPC-codes in the EMF can help to make a patient selection and to write a letter encouraging vaccination. The need of a reminder is essential, especially for patients at risk. Those who need a flu-shot the most, need to be stimulated the most. Together we can ensure high quality primary care with extra attention paid to prevention.



## Healthcare workers in Leicester, UK and their acceptance of pre-pandemic influenza vaccination

**Stephenson, Iain;** Dhillon, H.; Pareek, M.; Clarke, T.

University Hospitals Leicester, UK

**Background:** Influenza poses a hazard in hospitals with outbreaks increasing hospital stays and mortality. Healthcare workers (HCWs) are important reservoirs and, although vaccination reduces nosocomial spread, seasonal influenza vaccine uptake is low. Outbreaks of H5N1 have heightened pandemic concerns. During the first pandemic wave, HCWs will be susceptible thus significantly increasing nosocomial transmission. As availability of pandemic-specific vaccine will be limited, authorities have stockpiled H5N1 vaccine. We surveyed Leicester HCWs to identify factors associated with acceptance of pre-pandemic vaccination.

**Methods:** In February 2007, during an H5N1 outbreak at a Suffolk farm, a staff questionnaire was conducted at University Hospitals Leicester. This was repeated in August 2007 when there was no specific media coverage related to avian influenza. We aimed to sample 20% of 2500 employees with regular patient contact. Responses were analysed using descriptive statistics, nonparametric tests to compare vaccine “accepters” and “non-accepters”, and linear regression to determine which factors predicted acceptance of pre-pandemic vaccination.

**Results:** 520/525(99%) questionnaires were returned. Baseline characteristics of responders were similar in both periods. More health care workers would have pre-pandemic vaccination in February (166/262, 63%) than August (134/258, 52%;  $p=0.005$ ). Factors associated with accepting vaccine included: previous seasonal vaccine (OR 3.4,  $p<0.0001$ ), belief that seasonal vaccine benefits HCWs (OR 1.5,  $p=0.02$ ) and patients (OR 1.7,  $p=0.003$ ), awareness of seasonal vaccine campaigns (OR 1.6,  $p=0.009$ ), belief that pandemic risk is high (OR 22.5,  $p=0.002$ ), and that a pandemic threatens HCWs (OR 2.4,  $p<0.0001$ ). HCWs did not accept a vaccine if they believed pandemic risk was low (OR 0.21,  $p<0.0001$ ), and perceived the media overhyped “bird-flu” related issues (OR 0.32,  $p<0.0001$ ). Following multivariate linear regression, pre-pandemic vaccine acceptance was associated with previous seasonal vaccine ( $p<0.0001$ ), belief that seasonal vaccine benefits HCWs ( $p<0.0001$ ), belief that pandemic risk is high ( $p<0.0001$ ) and the belief that HCWs are at risk from the pandemic ( $p=0.001$ ). Reasons for non-vaccine uptake include: unconcerned about pandemic influenza (67%) and concerns over side effects (22%).

**Conclusion:** Achieving uptake of pre-pandemic vaccination is linked to improving seasonal vaccination programmes. Media reporting, but not overhyping, of avian influenza increases the likely uptake of HCW accepting pre-pandemic vaccination.

## Impact of influenza vaccination of nursing home staff on mortality among elderly residents. A cluster-randomized trial

**Lemaitre, Magali<sup>1</sup>**; Meret, M.T.<sup>2</sup>; Rothan-Tondeur, R.T.M.<sup>3</sup>; Belmin, B.J.<sup>4</sup>; Lejonc, L.J.L.<sup>4</sup>; Luquel, L.L.<sup>5</sup>; Piette, P.F.<sup>4</sup>; Salom, S.M.<sup>6</sup>; Verny, V.M.<sup>4</sup>; Vetel, V.J.M.<sup>7</sup>; Veyssier, V.P.<sup>8</sup>; Carrat, C.F.<sup>4</sup>

<sup>1</sup>UMR S707, France; <sup>2</sup>Résidence saint rémy, France; <sup>3</sup>ORIG, France; <sup>4</sup>APHP, France; <sup>5</sup>Service de gériatrie, Hôpital de Longjumeau, France; <sup>6</sup>Centre de gérontologie clinique, France; <sup>7</sup>Service de gériatrie, Le Mans, France; <sup>8</sup>Médecine Interne et Maladies Infectieuses, Compiègne, France

**Objective:** The influenza vaccination of staff in contact with immunocompromised persons, particularly the elderly, is strongly recommended, but its effectiveness is controversial. Our objective was to evaluate the impact of influenza vaccination of staff on all-cause mortality of institutionalized elderly people.

**Methods:** We conducted a cluster-randomized controlled trial in which 40 nursing homes matched in pairs were randomly allocated to a vaccination arm or a no-intervention control arm. Each institution was pair-matched for the following characteristics: size, staff vaccination coverage rate in 2005-2006 (0-20% or 20-40%), and an average disability score of residents. In the vaccination arm, influenza vaccination was voluntarily administered during a live interview. In the control arm, no intervention was provided. Residents over 60 years of age who were in the nursing home at the beginning of the study or who were admitted during the overall study period were included. The primary endpoint was total mortality of residents from two weeks before to two weeks after the influenza epidemic in the community. Secondary endpoints were hospitalizations and Acute Respiratory Illnesses (ARIs) in residents and the number of days with sick-leave from work in staff.

**Results:** Influenza vaccine coverage of staff was on average 68.8% (48.4-89.5%) in the vaccination arm vs. 31% (0-69.0%) in the control arm. The characteristics of elderly ( $n = 3483$ ) were similar between the two arms: mean age = 86 years, 77% are women, dependence GIR score = 2.9 and Barthel = 43, comorbidity index of Charlson = 2.3, 83% were vaccinated against influenza. A weak influenza epidemic was observed between January 15 and March 1, 2007. Primary analyses showed no significant reduction of mortality in the vaccination arm compared with the control arm (Odds-Ratio (OR)=0.86, 95% confidence interval (95%CI) 0.72;1.02 –  $P=0.08$ ). The difference was however significant in multivariate-adjusted secondary analyses (OR=0.80, 95% CI 0.66;0.96 –  $P=0.02$ ), and a strong correlation between vaccination coverage and mortality was observed (Spearman's  $r=-0.42$ ,  $P=0.007$ ). No significant reduction of hospitalizations was observed, while a difference in the rates of ARIs was noted (OR=0.62, 95%CI 0.57;0.69 –  $P=0.007$ ). Staff had more days of absence due to sick-leave in the control than in the vaccination arm.

## POSTER PRESENTATIONS

**Limitations:** Most of the differences between arms preceded the influenza epidemic period and coincided with peak activity of Respiratory Syncytial Viruses in the community.

**Conclusion:** The influenza vaccination for staff of nursing homes reduces the risk of acute respiratory illness among residents and sick-leave from work among staff. The decrease of 14% of all-cause mortality (non-significant), must be interpreted in the context of a weak influenza epidemic. Altogether, these results promote influenza vaccination of staff caring for the institutionalized elderly.

11-027

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### Influenza immunization of Dutch general practitioners: vaccination rate and reasons for (non)compliance

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**Van Essen, Ted<sup>1</sup>**; Opstelten, W.<sup>2</sup>; Ballieux, M.<sup>2</sup>; Goudswaard, A.N.<sup>2</sup>

<sup>1</sup>University Medical Center Utrecht, Netherlands; <sup>2</sup>Dutch College of General Practitioners, Netherlands

**Purpose:** The recommendation for yearly influenza vaccination to healthcare workers, including general practitioners, will be incorporated in the updated Dutch College of General Practitioners guidelines on influenza vaccination. For successful implementation, knowledge about the present vaccination rate and attitudes towards vaccination are indispensable.

**Design and methods:** In February 2008, we mailed a questionnaire to a random sample (n=730) of practicing general practitioners (GPs). Vaccination rate was determined and factors associated with non-compliance with vaccination were assessed using multivariable logistic regression. Reasons for (non-)compliance were registered.

**Results:** A total of 698 (96%) of the questionnaires were completed and returned. 248 GPs (36%) had been immunized against influenza. Independent factors related to non-compliance were female gender (adj. OR 0.5<sup>6</sup>; 95%-CI 0.38 to 0.82) and not working as a single-handed GP (0.6<sup>5</sup>; 0.43 to 0.99). Most frequent reasons for compliance were the protection against influenza (74%) and the lower risk of transmitting the influenza virus to patients (36%). Having no medical indication for influenza vaccination (52%) and the conviction of being protected against influenza by frequent professional exposure to the virus (28%) were the most frequent reasons for non-compliance.

**Conclusions:** The present influenza vaccination rate among Dutch general practitioners is rather low. Education about the effects of vaccination is needed to ensure their compliance with the recommendation of yearly influenza vaccination.

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**F. Hoffmann-La Roche Ltd**

Group Headquarters  
Grenzacherstrasse 124  
CH-4070 Basel  
Switzerland

Telephone +41-61-688 1111  
Telefax +41-61-691 9391

[www.roche.com](http://www.roche.com)



Combining innovation and expertise, Sanofi Pasteur MSD is the only company in Europe dedicated exclusively to vaccines. The company is a joint venture between sanofi pasteur, the vaccine division of sanofi-aventis, and Merck & Co. Inc.. Sanofi Pasteur MSD is able to draw on the research expertise of sanofi pasteur and Merck, together with their teams throughout the world, to focus on the development of new vaccines for Europe, which aim to extend protection to other diseases and perfect existing vaccines in order to improve the acceptability, efficacy and tolerability of vaccination.

- Company Name: **Sanofi Pasteur MSD**
- Company Address: 8 rue Jonas Salk
- Company Tel: +33 437284000
- Company Fax: +33 437284400
- Website: [www.spmsd.com](http://www.spmsd.com)

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Solvay Biologicals, a subsidiary company of Solvay Pharmaceuticals, has been producing influenza vaccines for more than 55 years and is one of the world's largest producers of influenza vaccines. The subunit vaccine Influvac® is marketed in over 65 countries worldwide. The production and development facilities are located in Weesp, the Netherlands. For a cell-based influenza vaccine significant investments in a new facility have been made. Solvay Biologicals plays an important role in the advancement of scientific knowledge on influenza and vaccination, through publications, scientific studies, sponsorships and other activities. Solvay Biologicals is one of the sponsors of the European Scientific Working group on Influenza (ESWI).

## **Solvay Biologicals**

PO Box 900, NL 1380 DA Weesp

Herman van Heemstra – Vice President Influenza Vaccines

T +31 294 477322

F +31 294 417772

E [influenza@solvay.com](mailto:influenza@solvay.com)

W [www.solvay-influenza.com](http://www.solvay-influenza.com)

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Baxter has developed a unique technology platform to manufacture Influenza vaccines at an industrial scale based on its proprietary serum protein free Vero cell technology, which can be used for the production of both interpandemic and pandemic vaccines such as H5N1.

At present, Baxter Vaccines markets 2 products, a vaccine against tick-borne encephalitis (FSME-IMMUN), as well as a group C meningococcal conjugate vaccine (NeisVac-C), and

distributes a second generation, smallpox vaccine (ACAM2000), which is licensed in the US by the FDA for government stockpiling.



Crucell N.V., headquartered in the Netherlands, is a global biopharma company focused on research, development, production and marketing of vaccines, proteins and antibodies that prevent and treat primarily infectious diseases. Crucell's core portfolio includes a vaccine against hepatitis B, a fully-liquid vaccine against five important childhood diseases, and a virosomal adjuvanted vaccine against influenza. Crucell also markets travel vaccines, such as the only oral anti-typhoid vaccine, an oral cholera vaccine and the only aluminium-free hepatitis A vaccine on the market. Crucell has a broad development pipeline, with several product candidates based on its unique PER. C6® production technology.

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Archimedesweg 4-6  
2333 CN Leiden  
The Netherlands  
Tel: +31(0) 71 519 91 00  
Fax: +31(0) 71 519 98 00  
[info@crucell.com](mailto:info@crucell.com)  
[www.crucell.com](http://www.crucell.com)



European Vaccine Manufacturers (EVM) is a specialized group within the European Federation of Pharmaceutical Industries and Associations (EFPIA), whose objective is to promote the benefits of vaccine and vaccination to public health. EVM members\* are European-based commercial organisations and major suppliers of vaccines in the world (total production: 4,7 billion doses) EVM mission is to:

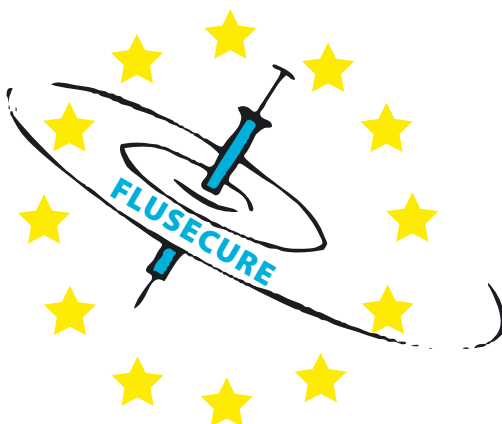
1. Create a supportive environment for improved vaccine protection and coverage;
2. Promote vaccine R & D to meet new challenges for innovative vaccine applications against infectious and other types of diseases;
3. Foster a favourable policy climate for the vaccine industry in Europe to bring new vaccines to the world.

\* Baxter, Crucell, GlaxoSmithKline Biologicals, MedImmune, Novartis Vaccines, sanofi pasteur, sanofi pasteur MSD, Solvay Biologicals, and Wyeth Vaccines

**European Vaccine Manufacturers (EVM)**

Rue du Trône 108 / Troonstraat 108 ·  
Leopold Plaza Building · B-1050 Brussels, Belgium  
Tel +32 2 626 25 55  
Fax +32 2 626 25 66

Magdalena R. de Azero  
Executive Director  
Tel.: 32.2.626.25.43  
[info@evm-vaccines.org](mailto:info@evm-vaccines.org)  
[www.evm-vaccines.org](http://www.evm-vaccines.org)



FLUSECURE is an EU-funded, not-for-profit project that brings together a consortium of public health institutions from 10 EU member states to facilitate the timely and sufficient production of pandemic influenza vaccines for the European population. To this end, the partners in FLUSECURE are developing:

- A tool set of pre-pandemic vaccine seed strains and supporting reagents
- Standardised immunological assays to predict protective immunity
- Pre-clinical testing and a multi-centre clinical trials network.

These reagents, technologies and services are available, through partnership agreements, to EU member governments and vaccine developers and manufacturers. These partnership opportunities will be promoted at the 3rd ESWI meeting.

**The FLUSECURE Consortium**

c/o Dr Amanda Semper  
Health Protection Agency  
Centre for Emergency Preparedness and Response  
Porton Down  
Wiltshire  
SP4 0JG

- Phone: 00 44 1980 616954
- Fax: 00 44 1980 610848
- Email: [flusecure@nvi-vaccin.nl](mailto:flusecure@nvi-vaccin.nl)
- Website: w



GSK Biologicals is a global vaccine company which has shown to be a leader in innovation. The company is active in the fields of vaccine research, development and production with over 30 vaccines approved for marketing and 20 more in development. Headquartered in Belgium, GSK Biologicals has 14 manufacturing sites strategically positioned around the globe. In 2007 GSK Biologicals distributed 1.1 billion doses of vaccines to 169 countries in both developed and the developing world – an average of 3 million doses a day.

GSK Biologicals employs over 9 000 people worldwide including more than 1 600 passionate scientists engaged in research aimed at discovering innovative vaccines that contribute to the health and well-being of people of all generations around the world.

**GlaxoSmithKline Biologicals**

89, Rue de l'Institut 1330 Rixensart  
Belgium

Tel: +32 2 656 8111

Fax: +32 2 656 8000

[www.gsk-bio.com](http://www.gsk-bio.com)



Novartis Vaccines and Diagnostics is a division of Novartis focused on the development of preventive treatments. The division has two businesses: Novartis Vaccines and Chiron. Novartis Vaccines is the world's fifth-largest vaccines manufacturer and second-largest supplier of flu vaccines in the US. The division's products also include meningococcal, pediatric and travel vaccines. Chiron, the blood testing and molecular diagnostics business, is dedicated to preventing the spread of infectious diseases through the development of novel blood-screening tools that protect the world's blood supply.

**Novartis Vaccines & Diagnostics**

350 Massachusetts Avenue

Cambridge, MA 02139

617-871-7000 main #

[www.novartis.com](http://www.novartis.com)

# sanofi pasteur

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Our vision is a world in which no one suffers or dies from a vaccine-preventable disease.

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- Company name **sanofi pasteur**
- Company address 2 avenue Pont Pasteur 69007 Lyon, France
- Contact name Hilda AGHAIKIAN
- Phone number + 33 4 37 37 70 95
- Fax number + 33 4 37 37 71 13
- Email [hilda.aghaikian@sanofipasteur.com](mailto:hilda.aghaikian@sanofipasteur.com)
- Website [www.sanofipasteur.com](http://www.sanofipasteur.com)



Quidel Corporation serves to enhance the health and well being of people around the globe through the discovery, development, manufacturing and marketing of rapid diagnostic solutions at the point of care in infectious diseases and reproductive health.

Marketed under the leading brand name of QuickVue®, Quidel's portfolio of products currently includes tests that aid in the diagnosis of several disease states, including influenza, RSV and Strep A. QuickVue products are sold to healthcare professionals through leading medical distribution partners on a worldwide basis with bioMérieux being the exclusive distributor for all countries outside of US, Japan and Scandinavia.

- Company name: **Quidel Corporation**
- Company address: 10165 McKellar Court,  
San Diego, California 92121 USA
- Contact name: Gigi Tzaferos
- Phone number : +858-646-8087
- Fax number : +858-552-6401
- Email : gtzaferos@quidel.com
- Website : www.quidel.com



A world leader in the field of in vitro diagnostics for 45 years, bioMérieux is present in more than 150 countries through 38 subsidiaries and a large network of distributors.

In 2007, revenues reached 1.063 billion euros.

bioMérieux provides diagnostic solutions (reagents, instruments, software), which determine the source of disease and contamination to improve patient health and ensure consumer safety. Its products are used for diagnosing infectious diseases and providing high medical value results for cardiovascular emergencies and cancer screening and monitoring. They are also used for detecting microorganisms in agri-food, pharmaceutical and cosmetic products.

- Company name: **bioMérieux SA**
- Company address: Chemin de l'Orme,  
69280 MARCY L'ÉTOILE, France
- Contact name: Pierre van Aarle
- Phone number : +33 4 78 87 20 00
- Fax number : +33 4 78 87 20 90
- Email : pierre.vanAarle@eu.biomerieux.com
- Website : www.biomerieux.com

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